THE EOSINOPHIL REGULATES IMMUNITY TO THE PARASITIC NEMATODE *TRICHINELLA SPIRALIS*

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THE EOSINOPHIL REGULATES IMMUNITY TO THE PARASITIC NEMATODE *TRICHINELLA SPIRALIS*

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Parasitic diseases pose a significant burden on the health of millions of people. Chronic helminth infections are typified by enhanced Th2 responses. Understanding how Th2 immunity is regulated is important for developing effective treatments for parasitic diseases and other Th2 disorders. Infection of mice with *Trichinella spiralis* is an especially useful model system to study the development of Th2 immunity to a natural pathogen. Eosinophilia is a prominent feature of *T. spiralis* infection, and we have shown previously that in eosinophil-ablated, transgenic mice, *T. spiralis* larvae die in large numbers in the muscle. Death is promoted by inducible nitric oxide synthase (iNOS). Neutrophils and $F4/80^+$ $CD11b^+$ $Ly6C^+$ macrophages produced iNOS.

Compared to fully developed muscle larvae, growing larvae were more susceptible to NO mediated killing *in vitro*. Larval growth was impaired in eosinophil-ablated mice, potentially extending the period of susceptibility to NO mediated killing. These changes were associated with reduced numbers of Th2 cells in infected muscle. Reduction in Th2 cell number was not caused by poor recruitment, but rather by impaired Th2 cell production in draining lymph nodes. Adding back eosinophils into ΔdblGATA recipients improved parasite growth and survival, as well as Th2 cell accumulation, supporting the role of the eosinophil in parasite retention and local immune regulation.

The influence of Th2 immunity on parasite growth was tested using mice deficient in STAT6 and IL-13. The results reveal that the IL-4/STAT6 axis is a key pathway that regulates parasite growth. Transcription of genes associated with nutrient deprivation was increased in muscles of infected eosinophil-deficient mice, but glycogen content of muscle larvae and muscle tissue was not altered by eosinophil deficiency. Taken together, the results show that eosinophils and STAT6 promote parasite establishment in the muscle by promoting larval growth while coincidentally inhibiting the Th1 immune response.

BIOGRAPHICAL SKETCH

Nebiat G. Gebreselassie was born and raised in Addis Ababa, Ethiopia. She completed high school at Nazareth Catholic School in Addis Ababa and moved to the US to pursue her bachelors degree in Biology and Chemistry from Salem College in Winston Salem, North Carolina. Nebiat joined the PhD program in the Graduate Field of Immunology in 2007.

DEDICATION

To my family and friends: Tsehai, Gebreegziabher, Genet, Tesfay, Julia, Isabell, Samerawit, Eli, Lewam, Million, Tigist, Leah, Deeqa, Hewan, Ichhya, Newal, George, Doe, Fr. Tom, Fr. Andrew, Fr. Anthony.

~ St. John, Arch. Michael and St. Anthony ~

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CHAPTER 1

Introduction[1]

[1] Sections of this chapter were taken from:

Appleton JA, Blum LK and Gebreselassie NG. Immunity to Parasitic Infection. In *Nematoda*, ed. Lamb TJ: Wiley-Blackwell (in press)

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Significance

Parasitic worms are complex organisms that are highly adapted to their hosts, commonly establishing chronic infections or demonstrating the capacity to repeatedly infect the same individual. Immune responses to parasitic helminths are characterized by expansion of Th2 populations and are associated with basophilia, eosinophilia, and alternative activation of macrophages [1]. Helminths employ a variety of evasive mechanisms that allow them to survive in their hosts for prolonged periods. Understanding pathways of immune evasion has significant implications in the development of novel therapeutic strategies against helminth infections. This dissertation describes a novel role of eosinophils and STAT6 in modulating Th2 immunity and parasite establishment during chronic muscle infection by *T. spiralis*.

Trichinella: classification, life cycle, and nurse cell formation

Classification

Trichinellosis is a zoonotic food-borne disease that is transmitted by ingestion of infected striated muscle tissue. *Trichinella* is a parasitic nematode of the family Trichinellidae, in the class Adenophorea. So far, seven species and five genotypes of *Trichinella* have been described from various species of birds, reptiles and mammals (Table 1) [2]. These are organized in to two clades based on genetic diversity that contribute to marked phenotypic variation (the presence or absence of a collagen capsule around the infected muscle cell). Other variations such as geographic and host localizations further distinguish *Trichinella* species [2].

Trichinella species*	Human infection	Host species	Geographical distribution
Encapsulated			
T. spiralis	+	Swine, rats, seldom in carnivores	Cosmopolitan
T. nativa	+	Terrestrial & marine carnivores	Holoarctic (arctic and subarctic)
T. britovi	+	Carnivores, seldom swine	Temperate Palearctic
T. murelli	+	Carnivores	Temperate Nearctic
T. nelsoni	+	Carnivores, seldom swine	Ethiopic region
Non-encapsulated			
T. pseudospiralis	+	Birds, mammals	Cosmopolitan
T. papuae	suspected	Swine, salt water crocodiles	Papua New Guinea, Thailand
T. zimbabwensis	unknown	Nile crocodiles, Nile monitor lizards, lion	Eastern Africa

Table 1.1. Species of *Trichinella*

*Genotypes T6, T8, T9, and T12 have been isolated from carnivores in North America, Southern Africa, Japan and Argentina, respectively. They have not been reported to cause disease in humans or to infect domestic animals.

Trichinella spiralis life cycle

The life cycle of *Trichinella* is completed in one host; there is no free-living stage. Infection is initiated when the host ingests meat that contains encapsulated *T. spiralis* L_1 larvae. Larvae are released from the muscle in the stomach and migrate to the intestines where they molt four times to become adults [2]. Adult *T. spiralis* worms reside in epithelial cells as intra-multi-cellular organisms for up to a maximum of three weeks until they are expelled by the host [2]. Fecund adult females release newborn larvae (NBL) in the small intestine. NBL enter the general circulation via mesenteric venules and become distributed throughout the body but they can only continue their life cycle in skeletal muscle cells [3, 4]. Parasite invasion induces a fully differentiated muscle cell to transform into one that supports the growth and development

of the larva [5]. This parasite-host cell complex (nurse cell complex) [6] is irreversible and can survive for as long as 30 years in humans and up to the lifetime of the organism in other mammals [2]. In rodents, the diaphragm and tongue are preferred sites of infection. Each larva invades a single myotube and completes its development in to an infectious L_1 in a course of 20 days [7, 8].

Biology of the nurse cell

The process of nurse cell formation is complex and many aspects of this cellular transformation are unknown. The larva induces several morphological and biochemical changes in the nurse cell. The infected cell, most likely in response to parasite secretions, down-regulates normal host gene transcription and up-regulates transcription of proteins that induce cellular transformation of the nurse cell such as vascular endothelial growth factor (VEGF) and collagens type IV and VI [9-12]. The nurse cell collagen capsule begins to form outside the infected cell starting 10 dpi and is completed in 20 days after muscle cell invasion [7, 13]. The nurse cell capsule is largely known to be composed of collagen types IV and VI [7, 12, 14]. However, the presence of other collagen types or extracellular matrix proteins have not been investigated. Larvae growth studies show that *T. spiralis* L_1 start growing as early as the first day of muscle infection. The parasites then remain dormant for 48h before resuming growth at a 39% growth rate until Day 19 [15] . This is supported by metabolic studies that show measurable increase in glucose uptake by parasites and/or infected muscle starting at eight days after muscle infection [16]. Using an *in vitro* system, it has also been shown that *T. spiralis* nurse cells transport significantly more glucose than uninfected skeletal muscle cells, suggesting that the

host supplements parasites with nutrients [17]. The nurse cell also induces the formation of a vascular network (circulatory rete) to presumably obtain nutrients and remove waste products [18, 19]. The circulatory rete is readily apparent around the nurse cell starting 12 dpi [20, 21].

Immune response during *T. spiralis* muscle infection

Muscle infection by *Trichinella* is characterised by the development of focal inflammation at the site of the infected muscle cell. Encapsulated species of *Trichinella* induce a more robust reaction when compared to non-encapsulated species [22]. The intensity of muscle inflammation is influenced by the intestinal phase of infection, as mice infected orally show greater myositis compared to mice infected by injections of NBL [23]. Cellular infiltrates include macrophages, eosinophils, neutrophils, and $CD4^+$ T cells, with few $CD8^+$ T and B lymphocytes [24, 25]. Macrophages are the most numerous cell type and are also observed in the cytoplasm of the nurse cell [26]. Myositis induced by *T. spiralis* is down-modulated in intensity as the parasite matures in the muscle [27].

T cells are critical in orchestrating leukocyte recruitment; infiltration does not occur in athymic mice [28]. Cytokine responses in draining lymph nodes are mixed initially, and then polarize to a Th2 phenotype (IL-4, IL-5, IL-10, and IL-13), which promotes a strong antibody response against tyvelose after 28 days of infection [23, 29]. Blood mononuclear cells recovered from human trichinellosis patients produce significant quantities of IFN-γ, IL-10, and IL-5, and retain the ability to proliferate in response to larval antigens for as long as 3 years after initial infection [30].

Nurse cells [22] are surrounded by alternatively activated (M2) macrophages, which are driven to an alternative phenotype by Th2 cytokines [31]. M2 macrophages appear to protect larvae, as classical activation of macrophages, and their production of NO, is associated with clearance of larvae from muscle [32, 33]. Alternatively activated macrophages are disseminated systemically in *T. spiralis*-infected mice, as evidenced by production of large quantities of Ym1 by peritoneal macrophages [34].

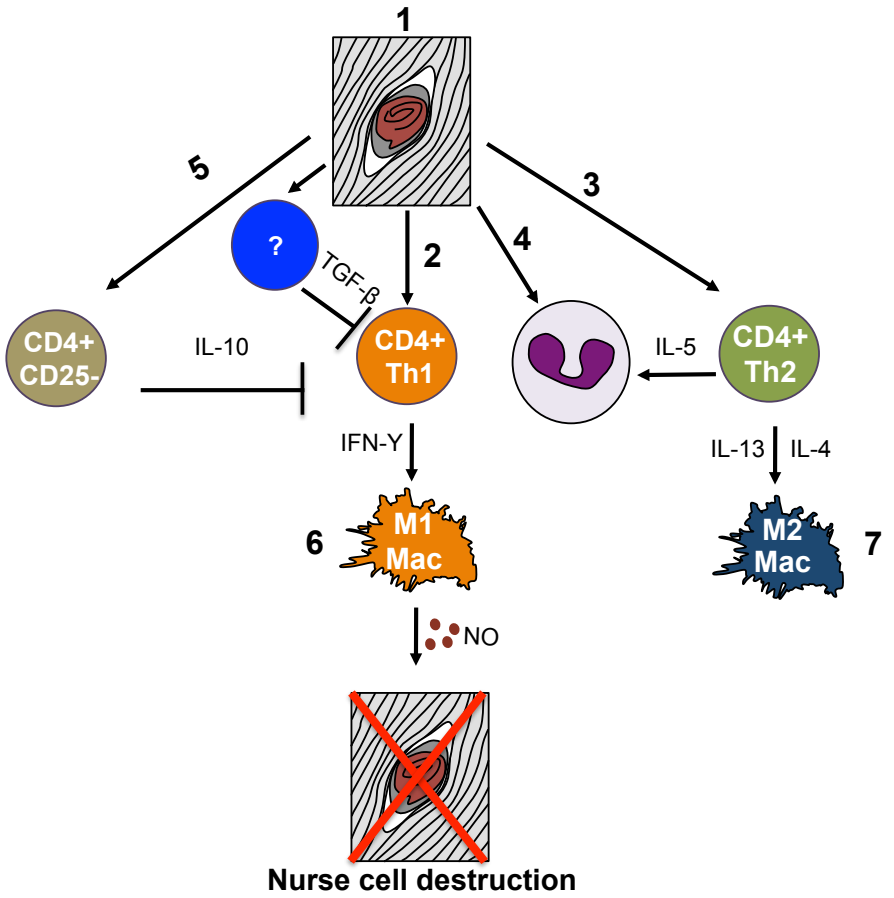

FIGURE 1.1. A model of immunity to *Trichinella* in the muscle.

Infection of skeletal muscle cells (1) induces a mixed Th1 (2) and Th2 (3) cellular response. Tissue eosinophilia (4) and the regulatory cytokines IL-10 and TGF-β (5) are also induced by infection and contribute to the control of classically activated M1 macrophages at sites of

infection (6), supporting a Th2 environment that promotes activation of alternatively activated M2 macrophages (7) and parasite survival.

Myositis is tightly regulated and generally does not lead to parasite destruction, which suggests that suppressive parasite or host factors are at work. IL-10 and TGF-β limit myositis and suppress IFN-γ and inducible nitric oxide synthase (iNOS) responses during the stage of infection when parasites are actively growing in the muscle (i.e., between 10 and 20 days post-oral infection) [23]. Muscle larvae burdens are moderately reduced in IL-10 deficient mice and larval survival improves with iNOS inhibition, implicating NO in parasite killing [35] [32]. Elimination of both TGF-β and IL-10 promotes an even more pronounced myositis, IFN-γ response, and parasite death (Figures 1.1). Once parasite growth inside the nurse cell is complete and chronic infection is established, the Th2 response dominates and coincidentally, regulation of inflammation switches to an IL-10 independent mechanism [23].

Eosinophils in immune regulation

Activated eosinophils are a heterogeneous population of cells, with variable granular, cytokine, chemokine, and surface phenotypes, compatible with a range of functional states. Eosinophils express MHC class II and have been shown to function as antigen presenting cells [36, 37]. Specifically, intraperitoneal injection of eosinophils that were pulsed with *Strongyloides stercoralis* antigen stimulated antigen-specific primed T cells to increase Th2 cytokine production when tested in restimulation assays [37]. Airway eosinophils have been shown to be capable of priming naïve T cells and stimulating $CD4^+$ T cells to produce Th2

cytokines[36]. The eosinophil derived neurotoxin in human eosinophils induces dendritic cell maturation via TLR2 activation, resulting in an expansion of the Th2 response [38].

In addition, eosinophils influence the recruitment of lymphocytes to sites of inflammation. In murine models of asthma, Th2 cytokine production is reduced in eosinophil-deficient mice, a result that derives from reduced T cell recruitment into the lung. Adoptive transfer of eosinophils or eosinophils and CD4 T cells reconstitutes disease in ΔdblGATA and PHIL mice, respectively [39, 40]. Transfer of eosinophils deficient in IL-13 failed to restore disease, documenting IL-13 as a critical mediator of the regulatory effect of eosinophils in allergic airway disease [39, 41]. Eosinophil deficient mice also show reduced expression of chemokines such as CCL7, CCL17, CCL22, and the eotaxins CCL11 and CCL24 in response to allergy challenge [39, 40]. Intranasal delivery of CCL11 restored T cell infiltration and Th2 cytokine responses [39] while blockade of CCL17 or CCL22 inhibited the recruitment of effector T cells [39, 40], implicating eosinophils in Th2 cell recruitment.

Eosinophils are present in certain healthy tissues, for example, the uterus, thymus and adipose tissue. IL-4 produced by eosinophils promotes alternative activation of macrophages in mouse adipose tissue, thereby promoting glucose tolerance and protecting against diet-induced obesity [42]. ΔdblGATA mice fail to support survival of plasma cells in the bone marrow, an effect that is attributed to a requirement for eosinophil-derived IL-6 and TNF- and APOL-related leukocyte expressed ligand 2 (APRIL) [43]. Thus, eosinophils are more than end stage effector cells and can function in physiologic and immunologic regulation.

Eosinophilia is a prominent feature of helminth infection and the contributions of eosinophils to host defense have been investigated *in vitro* and *in vivo*. IL-5 is the central

regulator of eosinophilia and several *in vivo* models have been developed to address the function of eosinophils by manipulating this cytokine at the gene and protein level. Evaluation of the influence of eosinophils in disease and parasite clearance has relied upon knock-out or overexpression of the gene encoding IL-5 , or depletion of the cytokine with specific antibodies. In other models, the gene encoding the receptor for IL-5 was disrupted or the receptor was blocked by antibody. Results of these studies must be interpreted with caution, as murine IL-5 has effects on cells other than eosinophils, most notably B lymphocytes [44]. With rare exceptions [45], the potential contribution of each of these IL-5 activities to the phenomena observed in IL-5 knock-out or transgenic mice has not been elucidated. Furthermore, IL-5 deficiency does not eliminate eosinophils entirely, rather it limits their proliferation, survival and recruitment to sites of infection. Other models employed to test the role of eosinophils are strains of mice engineered to be deficient in CC chemokine receptor 3 (CCR3) or eotaxin-1[46]. Both have defective eotaxin-mediated recruitment of eosinophils such that studies performed in these mice can address the requirement for eosinophils to enter sites of infection in order to affect parasite survival or immunity. Again, results must be interpreted with caution as CCR3 is expressed on cell types other than eosinophils, including mast cells, epithelial cells, and Th2 cells [47].

Investigations of the function of eosinophils have been aided significantly by development of two mouse strains in which the eosinophil lineage has been ablated. ΔdblGATA mice bear a deletion of the high-affinity double GATA site in the GATA-1 promoter[48]. In PHIL mice, a transgene encoding diphtheria toxin A was inserted downstream of the eosinophil peroxidase promoter [49]. Both constructs render mice free of eosinophils. These strains have

been tested for susceptibility and responsiveness to several parasitic helminths, in some instances yielding results that contrast dramatically with findings from mice deficient in or over-expressing IL-5. Two other strains in which genes encoding the granular proteins EPO and MBP have been knocked out are useful to test the significance of those eosinophil products [50, 51]. In the subsequent section, we will focus our attention on the results from experiments conducted in lineage-ablated and EPO or MBP knock-out mice. The findings provide evidence in support of eosinophil effector functions in parasite clearance in some instances, and evidence that the role of the eosinophil is negligible in others. In the case of *T. spiralis,* our findings support a regulatory role for eosinophils, as discussed in the following chapters.

Eosinophils in parasite infection

Dramatic results obtained in studies in which eosinophils were shown to adhere to helminth parasites, sometimes in the presence of antibody and/or complement, and to kill by discharging granules onto the parasite surface or releasing hydrogen peroxide *in vitro* have informed the widely held belief that eosinophils function as cytotoxic effectors in host defense against helminths [52]. Experimental evidence obtained in support of a role for eosinophils in protection and immune regulation *in vivo* is discussed below.

Eosinophils and tissue-dwelling helminths

The role of IL-5 in immunity has been investigated in a number of tissue-dwelling helminth infections. IL-5 is influential in resistance to infection with *Angiostrongylus cantonensis* [53, 54] *and S. ratti*, but is dispensable for immunity to *S. ratti* in secondary

infection[55]. The relationship of IL-5 deficiency to eosinophil function has yet to be clarified. IL-5 and CCR3 are critical for clearance of *Brugia pahangi* L3 in primary infections in the mouse model, although there does not appear to be a role for EPO or MBP in this immunity [56, 57]. Similarly, IL-5 and CCR3 are required for clearance of *Onchocerca volvulus* L3 from mouse skin, but EPO is dispensable [58]. The significance of these findings to natural infections with filarial worms is unclear, as the complete life cycles are not supported in the mouse. In mice infected with *L. sigmodontis*, deficiency in either MBP or EPO enhanced L3 establishment in association with reduced IL-4 [59]. Adult *L. sigmodontis* worms also developed faster in RAG-/-IL-4-/- mice, where eosinophilic responses were limited [60]. The findings are compatible with both effector and pro-Th2 regulatory roles for eosinophil products in *L. sigmodontis* infection. A similar role for eosinophils promoting protective Th2 responses is evident in *N. brasiliensis*-infected mice in which eosinophil ablation (ΔdblGATA) compromises development of immunity that limits the early tissue migratory larval stage during secondary infections[61]. The function of eosinophils in development of this immunity has not yet been determined.

More detailed understanding of regulatory and effector functions of eosinophils has been developed in a model of human *Strongyloides stercoralis* infection in which L3 are enclosed in a diffusion chamber prior to being implanted subcutaneously in mice. Experiments with mice deficient in CXCR2 or depleted of CCR3 revealed that innate immunity to *S. stercoralis* in this model involves direct killing by eosinophils and neutrophils [62]. Furthermore, it has been shown that the parasite synthesizes proteins, as well as chitin that are chemoattractants for eosinophils [63]. By producing these mediators, *S. stercoralis* would recruit eosinophils and engage their activity early in infection. Adaptive, protective immunity in this

model is dependent on a Th-2 response and production of IgM. In secondary infection, production of protective IgM is dependent on IL-5 and is restored when eosinophils are transferred to IL-5KO mice [45]. Independently, it has been reported that IgM induced by alum-adjuvanted antigen is compromised in ΔdblGATA mice and can be restored by transfer of eosinophils [36]. The relationship between enhancement of IgM production and antigen presentation by eosinophils [64] is unclear and the precise role of eosinophils in promoting IgM production remains to be elucidated.

Eosinophils and trichinellosis

Blood and tissue eosinophilia are pronounced during infection with *T. spiralis*. Although there are multiple reports that human eosinophils kill *T. spiralis* larvae *in vitro* [65-67], little or no alteration in parasite survival occurs in IL-5 deficient mice, IL-5 transgenic mice, or in mice depleted of eosinophils with anti-IL-5 antibodies [68-70]. In contrast, CCR3 KO mice are reported to sustain enhanced muscle larvae burdens following primary infection with *T.spiralis* [71] and IL-5 KO mice showed larger intestinal worm burdens and slower expulsion during secondary infection, two results that may implicate a host protective role for eosinophils [68].

Eosinophils constitute 10-15% of the infiltrating leukocytes at sites of muscle infection [29]. The functional consequence of eosinophil deficiency on *T. spiralis* muscle infection was tested using two mouse models of eosinophil ablation: PHIL and ΔdblGATA. *Trichinella spiralis* larvae die in large numbers in the absence of eosinophils, with reductions in muscle burdens ranging between 48 and 77% (Figures 1.2). Parasite death correlates with

enhanced IFN-γ and decreased IL-4 production in the draining lymph nodes. iNOS inhibition improved parasite survival, further implicating NO in parasite clearance. Increasing NO production by introducing IL-10 deficiency into the PHIL background dramatically enhances NO production and increases parasite killing to 90% or more. Thus, eosinophils regulate local immunity and protect the parasite during the muscle phase of infection. These findings are in stark contrast with those of *in vitro* studies that implicate the eosinophil as a mediator of *T. spiralis* destruction.

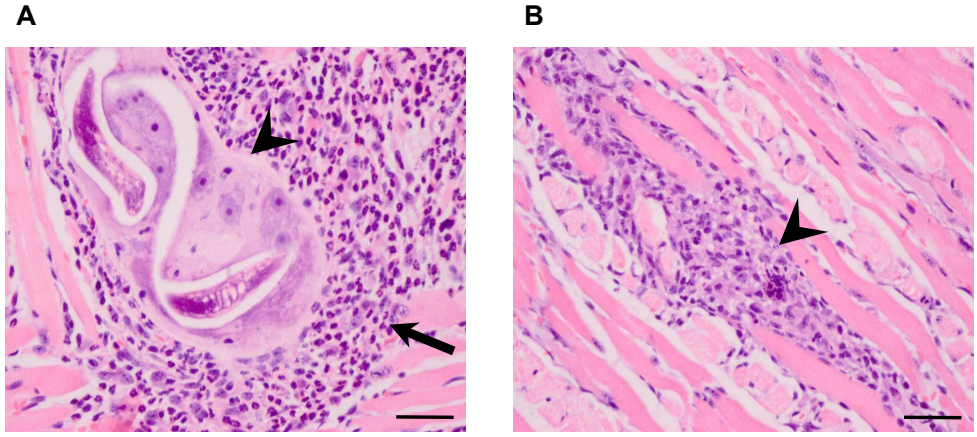

FIGURE 1.2. Photomicrographs of H&E stained sections of tongues from C57BL/6 and PHIL mice, 22 days post infection with *T. spiralis*.

***A*,** H&E stained section of tongue from infected C57BL/6 mouse, 22 days post infection. Leukocytes (arrow) surround the capsule of nurse cell (arrowheads). Larva is large and intact. ***B*,** In PHIL mice, nurse cell (arrowheads) is infiltrated and normal architecture is destroyed. Larva is not visible in the infiltrated nurse cell. Scale bar =50 μm.

The larvicidal effect of other oxidative radicals on different stages of *T. spiralis* larvae has also been evaluated *in vitro*. Comparison of the susceptibilities of different larval

stages indicated that *T.spiralis* NBL are most vulnerable to oxidative damage [72, 73] while expressed sequence tag clusters encoding antioxidants (thioredoxin oxidase, peroxiredoxin and glutathione peroxidase) were found in mature *T. spiralis* muscle larvae [74], suggesting that this life stage actively protects itself from reactive oxygen species (ROS). Nitric oxide has been implicated in helminth killing in other murine models, including *B. malayi* and *S. mansoni*. Treatment of mice with an inhibitor of NO synthase abrogates resistance to *B. malayi* [75, 76]. The parasites themselves are rich in cytoplasmic, cuticular and secreted anti-oxidant enzymes, including superoxide dismutases and glutathione peroxidase, which most likely help them survive in an unfavorable oxidative environment [77]. Studies conducted in mice infected with *S. mansoni* demonstrated that iNOS and NO production, induced by vaccination, participate in the reduction of worm burdens [78]. Endothelial cells and macrophages, stimulated *in vitro* to produce NO, can kill *S. mansoni* larvae [79] and susceptibility to the effects of NO has been shown to be larval age-dependent with older larvae showing greater susceptibility [80].

These studies suggest that ROS and RNS play important host defense roles against tissue-dwelling parasitic worms, in contrast to the well-established role for Th2 immune responses in expulsion of intestinal worms. Although increased oxidative radical production in these systems may successfully limit parasite numbers, it will also likely cause immune-mediated pathology. In *T. spiralis* infection, the eosinophil does not appear to be critical for initiation of a Th2 response in the intestinal phase of infection, but rather plays a crucial role in the maintenance of Th2 responses during the transition to the chronic phase, when the infection can be transmitted to another host. The contradictory findings between models of IL-5 deficiency and eosinophil ablation indicated that enhanced eosinophilia is not required for

regulatory impact to be manifest. Rather it appears that baseline, homeostatic numbers of eosinophils are essential. Parasite protection by eosinophils most likely benefits the host by preserving a Th2 response that prevents reinfection that may overburden the host as well as limiting injury to skeletal muscle. Challenge experiments in eosinophil-ablated mice will determine if eosinophils have host protective roles in secondary infections.

Synthesis

The results presented in this thesis describe the role of eosinophils and STAT6 in regulating immunity, parasite growth and survival during the muscle stage of infection with *T. spiralis*. We have previously shown that in eosinophil-ablated mice, *T. spiralis* muscle larvae die in large numbers and parasite death was caused by nitric oxide [81]. The studies reported in **Chapter 2** show that inflammatory macrophages and neutrophils that were recruited to sites of infection were sources of iNOS. In addition, parasite growth was compromised in eosinophil-ablated mice and this correlated with reduced Th2 cell accumulation at infection sites. Both Th2 cell accumulation and parasite growth/survival improved in eosinophil reconstituted mice, corroborating the regulatory role of the eosinophil during muscle infection.

Chapter 3 examines causes of Th2 cell number reduction and parasite growth inhibition. Results described in this section show that eosinophil deficiency dampens Th2 cell production in draining lymph nodes, which may subsequently impact Th2 cell accumulation at infection sites. In contrast to their function in allergic lung disease, eosinophils did not impact the recruitment potential or the migratory ability of T cells. Impaired parasite growth in eosinophil-deficient mice correlated with increased transcription of genes associated with nutrient deprivation. Our results also identify the IL-4/STAT6 signaling axis as the immune pathway that regulates parasite growth. IL-13 did not impact parasite growth or survival in the muscle. Finally, **Chapter 4** summarizes these results and discusses conclusions and future directions.

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CHAPTER 2

Eosinophils Preserve Parasitic Nematode Larvae by Regulating Local Immunity[2]

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Abstract

Eosinophils play important roles in regulation of cellular responses under conditions of homeostasis or infection. Intestinal infection with the parasitic nematode, *Trichinella spiralis*, induces a pronounced eosinophilia that coincides with establishment of larval stages in skeletal muscle. We have shown previously that in mouse strains in which the eosinophil lineage is ablated, large numbers of *T. spiralis* larvae are killed by NO, implicating the eosinophil as an immune regulator. In this report, we show that parasite death in eosinophil-ablated mice correlates with reduced recruitment of IL-4+ T cells and enhanced recruitment of inducible NO synthase (iNOS)-producing neutrophils to infected muscle, as well as increased iNOS in local F4/80+CD11b+Ly6C+ macrophages. Actively growing *T. spiralis* larvae were susceptible to killing by NO *in vitro*, whereas mature larvae were highly resistant. Growth of larvae was impaired in eosinophil-ablated mice, potentially extending the period of susceptibility to the effects of NO and enhancing parasite clearance. Transfer of eosinophils into eosinophil-ablated ΔdblGATA mice restored larval growth and survival. Regulation of immunity was not dependent upon eosinophil peroxidase or major basic protein 1 and did not correlate with activity of the IDO pathway. Our results suggest that eosinophils support parasite growth and survival by promoting accumulation of Th2 cells and preventing induction of iNOS in macrophages and neutrophils. These findings begin to define the cellular interactions that occur at an extra-intestinal site of nematode infection in which the eosinophil functions as a pivotal regulator of immunity.

Introduction

Investigations of infections caused by helminths that are natural parasites of rodents have revealed a number of mechanisms of protective immunity. Studies of the intestine-dwelling nematodes *Heligmosomoides bakeri*, *Nippostrongylus brasiliensis*, *Trichuris muris*, and *Trichinella spiralis* have documented Th2-driven immune responses that incorporate production of IL-4, IL-5, IL-9, IL-10, and IL-13, as well as basophilia, eosinophilia, and alternative activation of macrophages [1]. Parasite clearance from the intestine is abrogated in the absence of Stat6, IL-4, and/or IL-13, confirming the importance of these mediators; however, because of differences in habitats and life cycles, the specific effector mechanism that clears worms from the intestine varies among infections. For example, mast cells are crucial to expulsion of intraepithelial *T. spiralis* [2] but dispensable for clearance of *T. muris* and *N. brasiliensis* [3, 4] during primary infection. Among the cells that are prominent in immune responses to intestinal helminths, perhaps the most enigmatic is the eosinophil. Eosinophilia is a hallmark of nematode infection, yet infection of eosinophil-ablated mice with *T. muris*, *Schistosoma mansoni*, or *T. spiralis* has failed to reveal a key role for eosinophils in clearance of intestinal worms [5–7].

Immune responses and mechanisms of helminth clearance from extra-intestinal sites have been less thoroughly studied in natural rodent hosts. It has been shown that clearance of *Litomosoides sigmodontis* is promoted by the presence of eosinophil granular proteins, major basic protein 1 (MBP) and eosinophil peroxidase (EPO) [8]. Furthermore, eosinophils are necessary for development of immunity that limits the early tissue migratory larval stage during secondary infections by *N. brasiliensis* [9]. These findings support the paradigm of eosinophils as defenders against worm infection.

T. spiralis occupies both intestinal and extra-intestinal sites during the course of its life cycle Adult worms in the intestine release newborn larvae (NBL) that migrate to skeletal muscle and initiate chronic infection. Arrival of NBL in muscle is coincident with an intestinal Th2 immune response that expels adult worms and induces prominent blood and tissue eosinophilia [7]. Despite the magnitude of the local inflammatory response, intracellular muscle larvae mature to become infectious. We have shown previously that although eosinophil-ablated mice clear intestinal *T. spiralis* normally, immunity to the muscle stage of infection is impacted dramatically [7, 10]. Muscle larvae die in large numbers (50–75%) coincident with enhanced IFN-γ and decreased IL-4 production in draining lymph nodes. In the absence of eosinophils, leukocytes at sites of infection produce inducible NO synthase (iNOS) and parasite survival improves when mice are treated with specific iNOS inhibitors. Introducing IL-10 deficiency into the PHIL background dramatically enhanced NO production and increased parasite killing to $90%. These observations suggest that eosinophils protect developing larvae against NO-mediated killing [7].

In this study, we extend our earlier findings by showing that accumulation of IL-4+ T cells to sites of infection is reduced in eosinophil-ablated mice, and this correlates with infiltration of iNOS+ neutrophils and inflammatory macrophages during a time at which the growing larva is vulnerable to the effects of NO. Restoring eosinophils to infected mice improved Th2 cell recruitment, parasite growth, and survival, clearly implicating eosinophils as crucial to immune regulation that supports parasite survival.

Materials and Methods

Rats and mice

Adult Albino Oxford strain rats were produced and maintained in the Baker Institute Vivarium. IL-10-/-, PHIL, and ΔdblGATA (C57BL/6 background) [11] mice were bred at Cornell Transgenic Mouse Core Facility, and progeny were transferred to the Baker Institute. PHIL and IL-10-/- mice were genotyped as described previously [7, 12]. IL-5 transgenic, EPO-/-, and MBP-/- mice were described previously [12–14]. C57BL/6 ^{NHsd} mice (henceforth referred to as wild type [WT]) were purchased from Taconic. Animal care was in accordance with the guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care, and experiments were performed with the approval of the Institutional Animal Care and Use Committee of Cornell University.

Parasite and Ags

T. spiralis first-stage larvae (L_1) and NBL were recovered from rats as described previously (15, 16). For synchronous infection, 20,000 NBL suspended in serum-free DMEM (Mediatech) were delivered by retro-orbital injection. For oral infection, L_1 were suspended in 2% nutrient broth (Difco)-0.6% gelatin (Fisher Scientific), and doses of 300 L_1 were administered by gavage. Mice were euthanized by CO_2 inhalation at the times indicated in each experiment. Muscle larvae burdens were assessed 24 days post-infection (dpi) or later in whole carcasses as described previously [15]. In some experiments, larvae were recovered from diaphragms 12–18 dpi by digesting minced tissue for 15 min at 37˚C in 5 mg/ml collagenase I (Sigma). Somatic Ags from L_1 were prepared as described previously [17].

Eosinophil transfer experiments

Eosinophils were obtained from the peritoneum (by lavage) and spleens (SPLs) of infected IL-5 transgenic mice 12–20 dpi. Cells were pooled and purified by either positive or negative MACS bead selection. For positive selection, eosinophils were enriched using PE-conjugated anti–Siglec-F Ab (BD) and anti-PE microbeads (Miltenyi Biotec), a procedure that yielded eosinophil preparations of 93% purity. For negative selection, contaminating cells were labeled with PE-conjugated rat anti-mouse CD90.2, B220, NK1.1, CD11c, F4/80, Ly-6G, and Ly-6C Abs (eBioscience) and anti-PE microbeads (Miltenyi Biotec), yielding preparations that were 83% eosinophils. After washing twice in PBS, $5x10^6$ purified cells were resuspended in 200 ml sterile PBS and injected i.v. into ΔdblGATA mice on alternative days for 10 d, as indicated in Fig 2.7A. Transfer of cells recovered by the two methods yielded similar results.

Histology and immunohistochemistry

Histochemical staining and immunohistochemistry were performed as described previously [10]. Leukocytes were recovered from diaphragms and cells were prepared for cytologic staining as previously described [15]. Slides were stained with rabbit polyclonal anti-iNOS (NeoMarkers) and hematoxylin (Fisher), and differential counts were performed under 40X magnification using a BX51 microscope (Olympus).

Cytokine ELISA

Cells from cervical lymph nodes (CLNs) were obtained and cultured as described previously (10). IL-4, IL-5, IL-10, IL-13, and IFN-γ were assayed in culture supernatants by ELISA as described previously [7].

Flow cytometry

Cells were recovered from individual diaphragms as described previously [10]. For intracellular IL-4 detection, cells were cultured *ex vivo* for 5 h with 250 ng/ml ionomycin (Sigma-Aldrich), 50 ng/ml PMA (Sigma- Aldrich), and 1 mg/ml brefeldin A (BD Pharmingen). After a 15-min incubation with Fc block (eBioscience) and 10% normal mouse serum, cells were incubated for 15 min with FITC-conjugated anti-CD8 and PE-Cy7– conjugated anti-CD4 (eBioscience). Samples were treated with fixation/ permeabilization buffer (eBioscience), and permeabilized cells were stained using PE-conjugated anti–IFN-γ and allophycocyanin-conjugated anti– IL-4 (eBioscience). For intracellular iNOS detection, permeabilized diaphragm leukocytes were incubated with a rabbit polyclonal anti-iNOS (NeoMarkers) followed by allophycocyanin-conjugated goat anti-rabbit (IMGENEX). Cells were first stained for cell surface Ags as described above using PE-conjugated anti-CD11b, PE-Cy7–conjugated anti-F4/80, and FITC-conjugated anti–Ly-6C (eBioscience). For basophil counts, cells isolated from CLN, mesenteric lymph node (MLN), and SPL were stained with FITC-conjugated anti-CD49b (BioLegend), PE-conjugated anti–FcεR1-α (BioLegend), and allophycocyanin-conjugated anti–c-Kit (BioLegend).

Parasite measurements and susceptibility to NO Developing L_1 were recovered by digesting minced diaphragms for 15 min at 37℃ in 5 mg/ml collagenase I (Sigma). To prevent curling, we treated larvae with 70% ethanol at 56℃ and left them overnight at room temperature. Straightened larvae were centrifuged and resuspended in 5% glycerol/70% ethanol to soften and clear them before preparation for cytospin. The cytospin slides were stained with HEMA-3 (Fisher Health- care), and measurements were performed using 103 and 203 objectives on a

BX51 microscope (Olympus) by fitting a polygon around the boundary of the larva and computing the area (Microsuite Basic Olympus software). At least 20 larva were measured per mouse, and values are expressed in micrometers squared. To evaluate susceptibility of larvae to NO-mediated killing, developing L_1 recovered from synchronously infected WT mice at 8, 11, 14, and 21 d post-injection or NBL were cultured with indicated concentrations of (Z)-1-[N-(2-aminoethyl)-N-(2-ammonioethyl)amino]dia- zen-1-ium-1, 2-diolate (diethylenetriamine [DETA] NONOate) (Cayman Chemical) or DETA (Sigma) for 48 h (37˚C, 8% CO2) in DMEM (Mediatech) supplemented with 30% fetal bovine serum (Gibco). Parasite death (larvae that were immobile and granular) is reported as a percentage of the total.

Quantitative RT-PCR

Total RNA was isolated from diaphragm tissue using TRIzol reagent (Invitrogen), and cDNA was prepared using SuperScript III First-Strand cDNA Synthesis System (Invitrogen). A sample lacking reverse transcriptase served as negative control. Quantitative RT-PCR (qRT-PCR) was performed for NOS2, ARG1, FIZZ-1, YM1, and GAPDH using the following TaqMan Gene Expression primers and probes: GAPDH: forward, 59-TGTCAAGCTCATTTCCTGGTATGA-39; reverse, 59-CTTACTCCTTGGAGGCCATGTAG-39; probe, 59-TCCACCACCCTGTTGCTGTAGCCG-39; YM1: forward, 59-TTTGCTGGAATGCAGAATAATGAG-39; reverse, 59-CAATGCTTCATAGTCACGCAAGT-39; probe, 59-TCACTTACACACATGAGCA-39; ARG1: forward, 59-AACGGGAGGGTAACCATAAGC- 39; reverse, 59-TGATGCCCCAGATGGTTTTC-39; probe, 59-ACTGACTACCTTAAACCAC-39; NOS2: forward, 59-CAGCTGGGCTGTACAAACCTTT-39; reverse, 59-

CATTGGAAGTGAAGCGTTTCG-39; probe, 59- CGGGCAGCCTGTGAGACCTTTGA-39; FIZZ1: forward, 59-TCCAGCTAACTATCCCTCCACTGT-39; reverse, 59-GGCCCATCTGTTCATAGTCTTGA-39; probe, 59-CGAAGACTCTCTCTTGCT-39. Primers and probes for IDO were purchased from Applied Biosystems. qRT-PCRs were performed using the ABI PRISM 7500 Sequence Detection System and its analysis software, SDS 2.3 and RQ Manager (PE Applied Biosystems).

Measurement of IDO activity

IDO activity was measured by quantifying kynurenine (KYN) in culture supernatants of CLN and diaphragm cells that were recovered from infected WT, PHIL, and ΔdblGATA mice and cultured as described previously [10].

Statistical analysis

All experiments were performed two to four times with similar results. Means ± SD were calculated from data collected from individual mice unless otherwise indicated. Significant differences were determined using Student t test or ANOVA with Tukey's post hoc test for multiple means. Statistical analysis was performed with GraphPad Prism 4 software.

Results

Cellular sources of iNOS in eosinophil-ablated mice

We have reported previously that iNOS contributes to clearance of muscle larvae in eosinophil-ablated mice [7]. To better characterize the destructive immune response, we used qRT-PCR to document phenotypic changes in local macrophages. We analyzed the expression of genes associated with alternatively activated (M2) macrophages (ARG1, YM1, and FIZZ1) and

classically activated (M1) macrophages (NOS2) in diaphragms of infected WT, ΔdblGATA , and PHIL mice. Although all three strains dramatically upregulated M2 markers, eosinophil ablation was associated with a marked increase in NOS2 transcription and decreases in M2 marker expression at 17 dpi (Fig. 2.1A). Immunohistological analysis of leukocytes recovered from diaphragms confirmed the presence of iNOS+ macrophages but also revealed large numbers of iNOS+ neutrophils in PHIL mice (Fig. 2.1B). Although the representation of iNOS+ cells among neutrophils was markedly greater than that of macrophages, the number of each cell type in infected muscle [7] is such that iNOS+ macrophages outnumbered neutrophils in diaphragms of eosinophil-ablated mice by a ratio of 2:1. Overall, macrophage accumulation was not affected by eosinophil ablation (Fig. 2.1C), but among phenotypically distinct macrophage subsets, Ly-6C+ CD11b+ F4/80+ inflammatory macrophages produced significantly more iNOS in PHIL mice compared with WT (Fig. 2.1D).

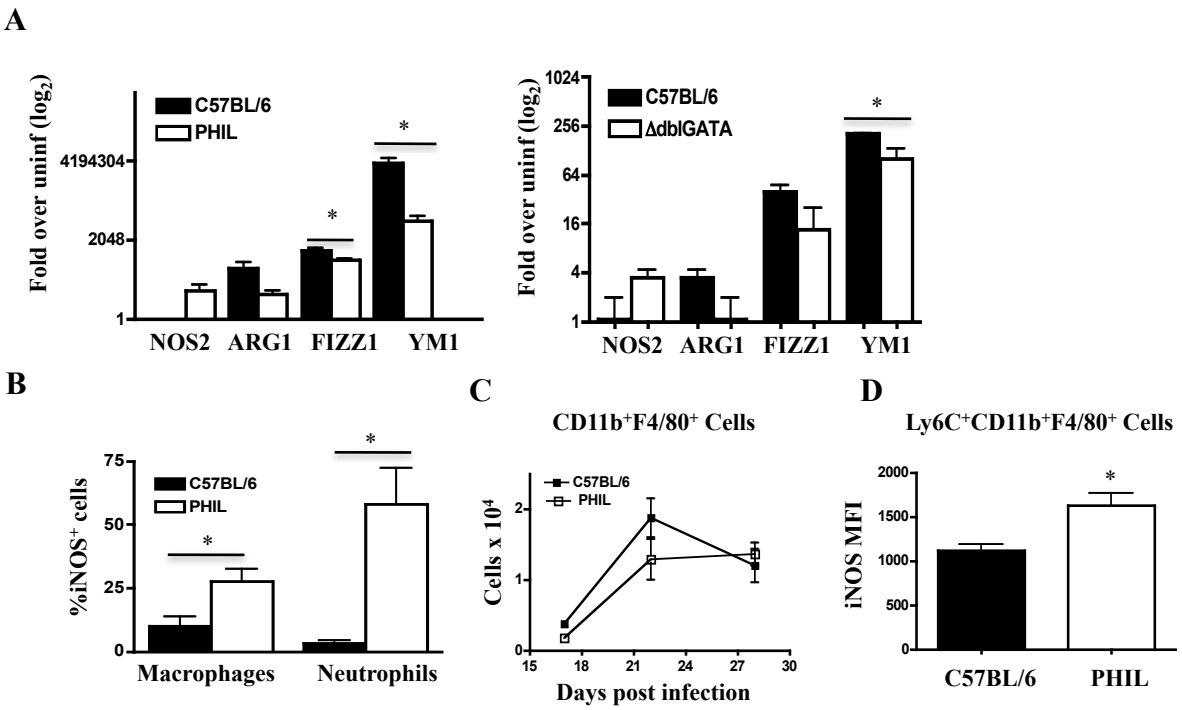

FIGURE 2.1. Cellular sources of NOS2 in eosinophil-deficient mice.

A, qRT-PCR results for selected macrophage markers in diaphragms of PHIL, ΔdblGATA, and WT mice 17 dpi. *B*, Identification of iNOS+ cells by immunohistochemistry in cytospin preparations of diaphragm-infiltrating cells collected 22 dpi. *C*, Numbers of F4/80+CD11b+ macrophages in diaphragm leukocytes recovered from WT and PHIL mice. Cells were phenotyped by flow cytometry. *D*, Mean fluorescence intensity for iNOS among Ly-6C+ CD11b + F4/80+ inflammatory macrophages in PHIL and WT mice at 22 dpi. Experiments were performed two to four times with similar results. Values represent means ± SD; n = 3–6 mice. Significant differences were determined by Student t test, *p , 0.05.

Impact of eosinophil deficiency on leukocyte recruitment

Histologic examination of tongues revealed that the cellular infiltrates around nurse cells in PHIL mice were reduced compared with WT (Fig. 2.2A). Enumeration of leukocytes recovered from diaphragms confirmed a reduction in absolute cell numbers at sites of infection in PHIL mice (Fig. 2.2B). Flow cytometric evaluation of T cell subsets documented fewer CD4+ and CD8+ T cells (Fig. 2.2C), as well as significantly reduced frequencies of IL-4+ CD4+ and IL-4+ CD8+ T cells in diaphragms of PHIL mice (Fig. 2.2D, E). The results suggest that eosinophils promote local recruitment of IL-4–producing T cells.

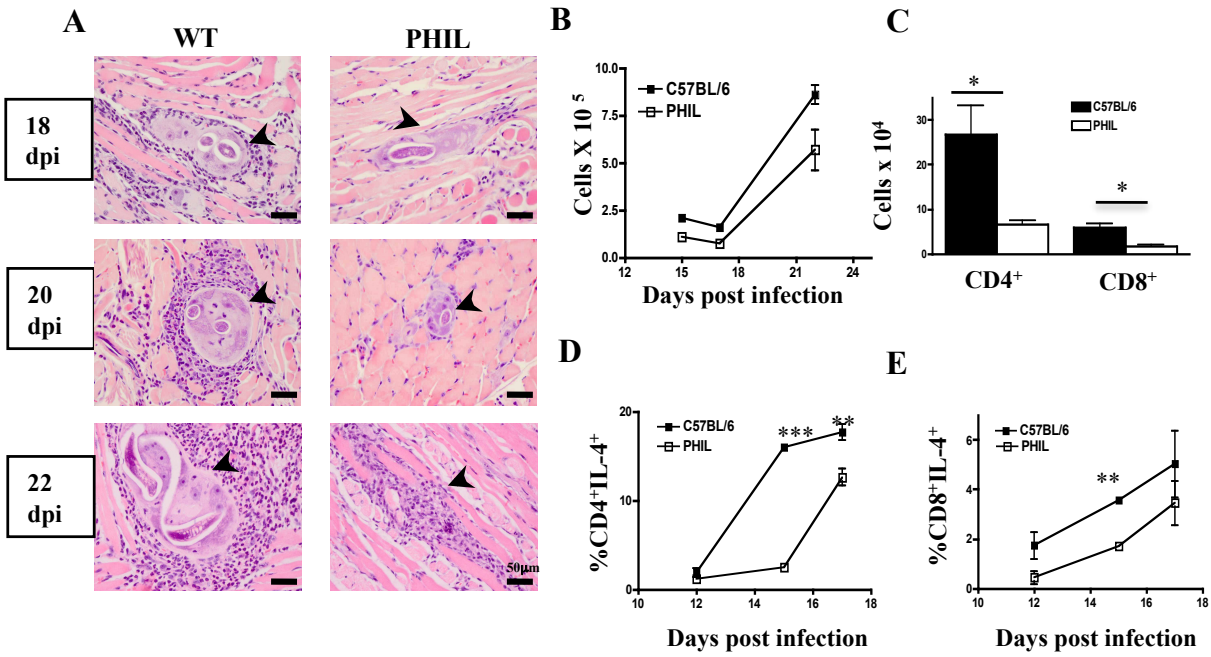

FIGURE 2.2. Leukocyte accumulation and T cell responses in the absence of eosinophils. *A*, H&E-stained sections of tongues collected from infected WT and PHIL mice. Arrowheads indicate nurse cells. Scale bars, 50 μm. *B*, Number of leukocytes recovered from diaphragms of infected PHIL and WT mice. *C*, Numbers of CD4+ and CD8+ T cells recovered from

diaphragms of infected PHIL and WT mice at 15 dpi and *(D, E)* frequency of IL-4 production by these cells 12–17 dpi. Surface phenotype and cytoplasmic cytokines were assayed by flow cytometry. Experiments were performed two or three times with similar results. Values represent means ± SD; n = 3–4 mice. Significant differences were determined by Student t test. *p , 0.05, **p , 0.001, ***p , 0.0001.

Potential for IDO to regulate T cell responses

IDO mediates oxidation of tryptophan to KYN and has been implicated in regulating Th1 responses. Human eosinophils produce IDO in response to IFN-γ, thereby promoting apoptosis or inhibiting cellular proliferation of Th1 cells [18]. We investigated whether IDO might be the link between eosinophils and reduced Th1 responses in WT mice by investigating gene expression and enzyme activity during infection. IDO gene expression was similar in infected muscles of PHIL, ΔdblGATA , and WT mice (Fig. 2.3A). IDO enzymatic activity, measured by KYN production in cultures of Ag-stimulated CLN cells or diaphragm leukocytes, was similar across strains (Fig. 2.3B). Immunohistochemical staining of diaphragm leukocytes showed that the percentages of IDO+ cells were similar between WT and PHIL mice (Fig. 2.3C). Large mononuclear cells, but not eosinophils, were IDO+ in tissues of WT mice. The results do not support a role for IDO in eosinophil-dependent regulation of local T cell responses.

Impact of eosinophil deficiency on basophilia

We examined the impact of eosinophil ablation on basophilia by counting basophils in MLN, CLN, and SPL at 0, 2, 5, and 12 dpi (Fig. 2.3D). Uninfected ΔdblGATA mice had significantly fewer basophils in the SPL, and this trend was evident in the MLN, CLN, and SPL on 2 and 5 dpi, although differences were statistically significant only in the SPL at 2 dpi. Numbers of basophils in all tissues were similar in the two mouse strains on 12 dpi, before the time at which parasite compromise and altered immunity are evident in eosinophil-ablated mice.

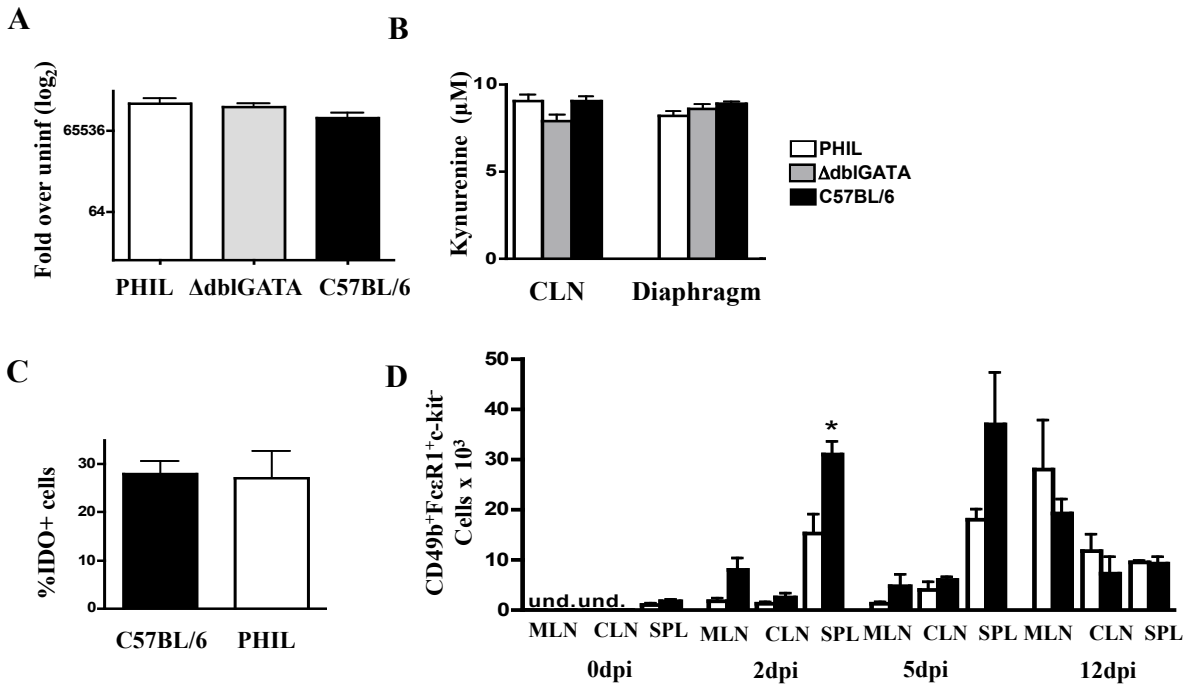

FIGURE 2.3. IDO response and basophilia in eosinophil-ablated mice.

A, IDO gene expression in diaphragms of infected ΔdblGATA, PHIL, and WT mice 15 dpi. *B*, KYN in cultures of CLN and diaphragm leukocytes collected 15 dpi. *C*, Detection of IDO in

diaphragm leukocytes from infected WT and PHIL mice collected 12 dpi. *D*, Basophils in WT and ΔdblGATA lymphoid tissues. Basophils were identified as CD49b+ FcεR1+c-Kit2 cells in the CLN, MLN, and SPL in uninfected and infected mice 2, 5, and 12 dpi by flow cytometry. Experiments were performed two times with similar results. Values represent means ± SD; n = 3–4 mice. Significant differences were determined by Student t test. *p , 0.05.

Eosinophil granular proteins do not influence survival of muscle larvae

We investigated the impact of eosinophil granular proteins on the progression of infection and modulation of immune responses by infecting mice deficient in either EPO or MBP. Muscle larvae burdens in both strains were similar to WT (Fig. 2.4A). Cytokine production by CLN cells in response to Ag re-stimulation was not altered (Fig. 2.4B), nor was there an effect on the numbers of eosinophils, CD4+ cells, or CD8+ cells at sites of infection (Fig. 2.4C). Thus, MBP or EPO were dispensable for regulation of T cell responses and parasite survival.

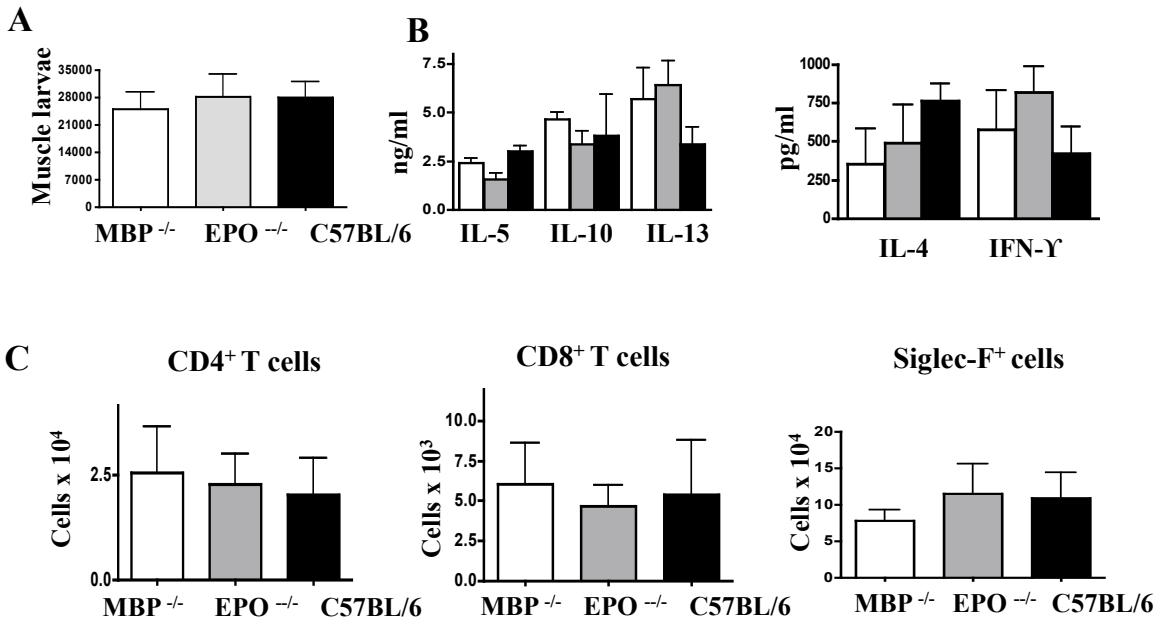

FIGURE 2.4. Effects of MBP and EPO on parasite survival and immunity.

A, Larval burdens in muscles of WT, EPO -/-, and MBP-/- mice 28 dpi. *B,* Cytokines measured in Ag-stimulated cultures of CLN cells collected from WT, MBP -/-, and EPO -/- mice 17 dpi. *C*, CD4+, CD8+, and Siglec-F+ cells recovered from diaphragms of infected WT, MBP -/-, and EPO -/- mice 17 dpi. Experiments were performed twice with similar results. Values represent means ± SD; n = 3–4 mice. No significant differences were found.

Larval growth is impaired in the absence of eosinophils

Microscopic examination of H&E-stained tongue sections revealed that both nurse cells and larvae appeared to be smaller in PHIL versus WT mice (Fig. 2.2A). To determine whether larval growth was compromised in the absence of eosinophils, we measured the dimensions of larvae recovered from diaphragms between 12 and 18 dpi. The mean area of

larvae was similar in PHIL versus WT mice on 12 dpi but was reduced in PHIL mice on 15 and 18 dpi (Fig. 2.5A). Similarly, a significant difference in larval area was detected in ΔdblGATA versus WT mice on 17 dpi (Fig. 2.5B). Larvae grew normally in MBP-/- and EPO-/- mice (Fig. 2.5C). To test whether iNOS activity, in the presence of eosinophils, would cause impaired larval growth, we infected IL-10-/- mice. This strain has dramatic, local iNOS+ cellular infiltrates and some reduction in larval burden [7, 10]. Parasite growth was not altered by IL-10 deficiency (Fig. 2.5D), indicating that nitrosative stress does not compromise parasite growth.

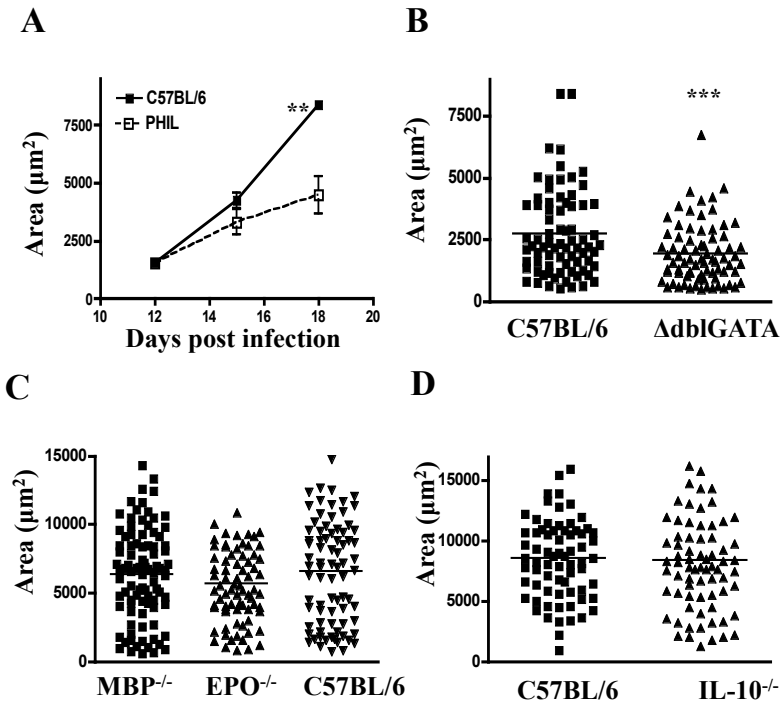

FIGURE 2.5. Larval growth in eosinophil-ablated mice.

Estimated area of larvae recovered from *(A)* PHIL and WT mice between 12 and 18 dpi, *(B)* ΔdblGATA and WT mice 17 dpi, *(C)* EPO -/-, MBP -/-, and WT mice 18 dpi, and *(D)* IL-10 -/- and WT mice 18 dpi. A total of 25–30 larvae were evaluated per mouse. Values represent means

± SD; n = 3–4 mice *(A)*. Bars indicate the mean values from 75–90 larvae pooled from three mice *(B–D)*. Experiments were performed two to four times with similar results. Significant differences were determined by Student t test *(A, B, D)* or by ANOVA and Tukey's test *(C)*. **p , 0.001, ***p , 0.0001.

Developing *T. spiralis* larvae are susceptible to direct killing by NO

To determine whether death of larvae is the direct result of exposure to NO, we tested the larvicidal effect of NO on *T. spiralis* larvae *in vitro*. Larvae that recovered from WT mice at different times post-infection were cultured for 48 h with the artificial NO donor (DETA-NONOate or with the vehicle control DETA. To generate larvae that were at the same stage of development, we infected mice by i.v. injection of NBL to achieve synchronous muscle infections. The results show that NBL and muscle larvae up to 11 d of age were killed in significant numbers (Fig. 2.6A, B), whereas relatively mature muscle larvae (14 d old) were resistant (Fig. 2.6C). Note that because of the asynchronous nature of NBL production by adult worms in the intestine, NBL colonize the muscle between 4 and 14 d after oral infection [19]. Thus, susceptible larvae would be present in the muscle between 4 and 28 d after oral infection, with the proportion of susceptible larvae declining between 14 and 28 d. In support of the conclusion that only growing muscle larvae are susceptible to the effect of NO, we found that parasite clearance in PHIL mice is completed by 28 dpi, with no additional reduction in burden evident when estimated 40 dpi (Fig. 2.6D).

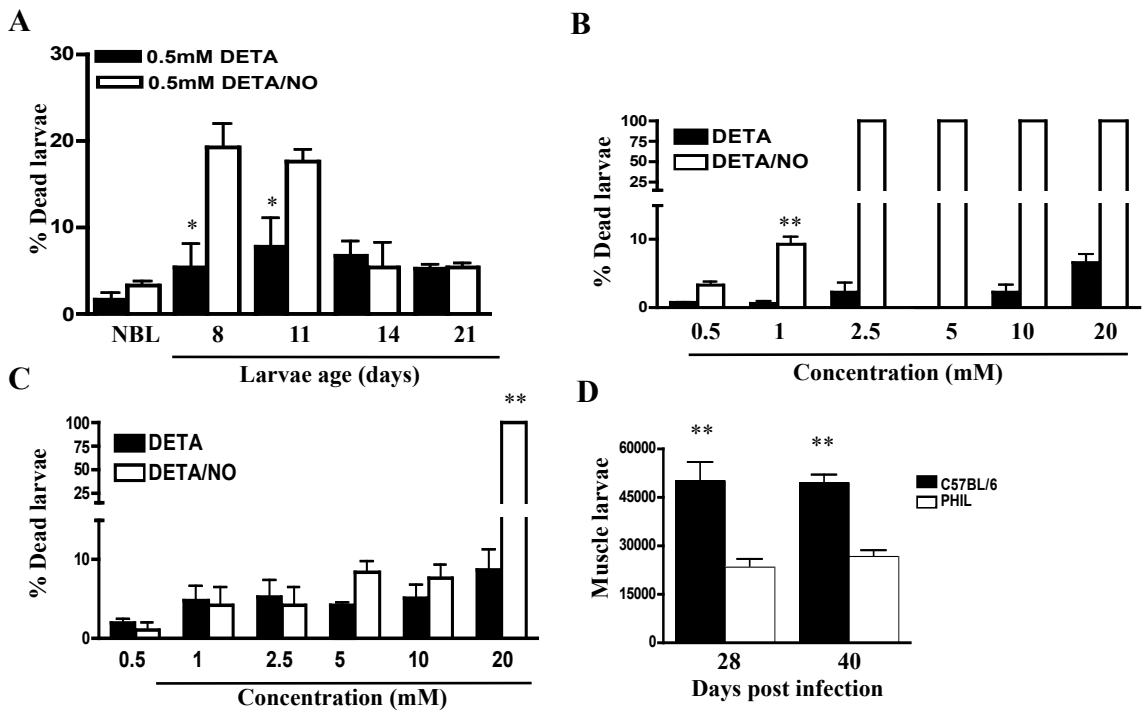

FIGURE 2.6. Susceptibility of muscle larvae to NO-mediated killing.

Susceptibilities of different larval stages of *T. spiralis* to NO-mediated killing were determined by culturing *(A)* 8- to 14-d-old larvae isolated from C57BL/6 mice, *(B)* NBL, or *(C)* mature L_1 (24 d old) with DETA/NO or DETA for 48 h *in vitro*. Viability was estimated for 60 larvae/well, three wells per mouse, mean ± SD for three mice per time point. *D,* Muscle burdens in PHIL and WT mice, 28 and 40 dpi. Experiments were performed twice with similar results. Values represent means ± SD; n = 3–4 mice. Significant differences were determined by Student t test. *p , 0.05, **p , 0.001.

Adoptive transfer of eosinophils improved larvae growth and survival

To confirm the role of the eosinophil in parasite growth and retention, we transferred eosinophils isolated from infected IL-5 transgenic mice to infected ΔdblGATA mice (Fig. 2.7A). Based on results of replicate experiments showing that transferred eosinophils extravasated in skeletal muscle and persisted there for 24 h but not 48 h (not shown), we designed the experimental protocol to incorporate cell transfers on alternate days between 5 and 15 dpi. Four experiments, conducted using positively selected (n = 2) and negatively selected (n = 2) eosinophils, yielded similar results, and statistical analysis documented that the improvement in larval burdens in this group of experiments averaged 36% and was highly significant ($p = 0.005$; Fig. 2.7B). One experiment performed with PHIL mice showed that transfer of positively selected eosinophils improved larval burdens by 42% ($p = 0.04$). Results from a single experiment performed with negatively selected eosinophils in ΔdblGATA mice (Fig. 2.7C–E) show that transfer of eosinophils improved larval burdens in the diaphragms of recipients (Fig. 2.7C) and promoted a modest but significant enhancement of larval growth (Fig. 2.7D). Although cytokine responses in recall assays performed with CLN cells were not altered by eosinophil transfer (Fig. 2.7E), Th2 accumulation at sites of infection improved markedly when eosinophils were transferred to ΔdblGATA mice (Fig. 2.7F).

Figure 7

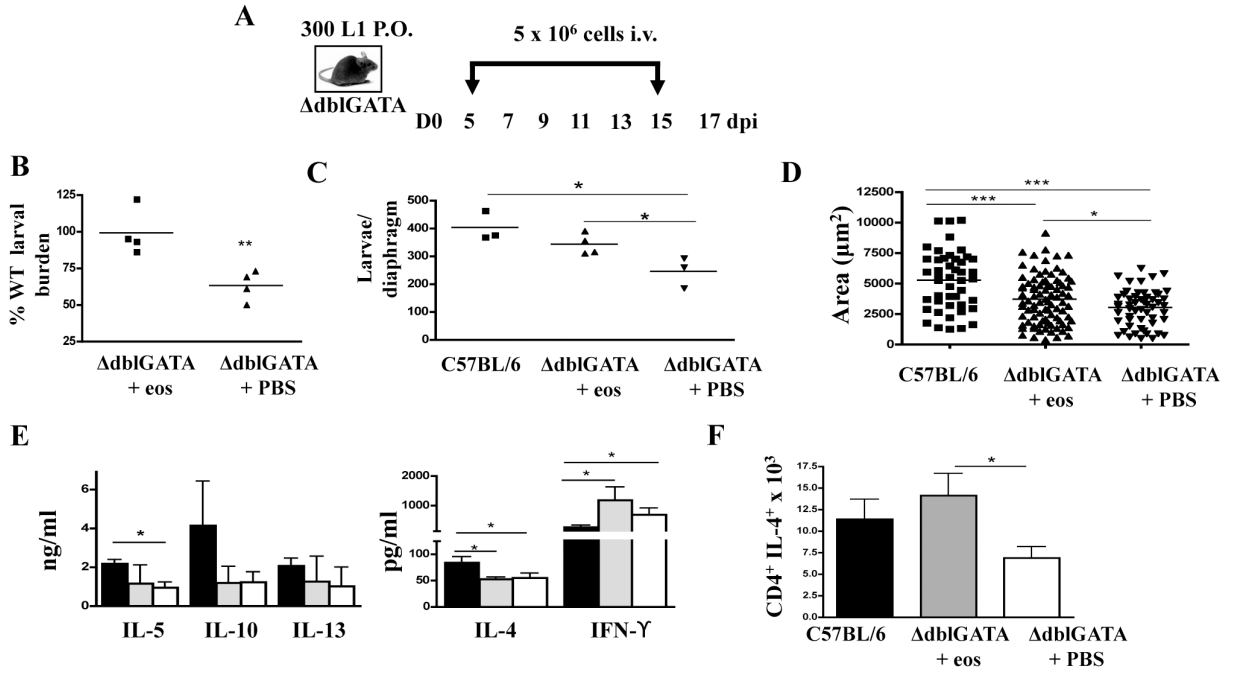

FIGURE 2.7. Influence of eosinophil transfer on larval growth and survival.

A, Design of experiments in which ΔdblGATA mice were given 5 x 10^6 eosinophils or PBS every 48 h between 5 and 15 dpi. *B*, Parasite survival in ΔdblGATA mice after transfer of eosinophils (prepared by positive or negative selection) or PBS. Values are expressed as a percentage of the burden in control C57BL/6 mice. Each point is a mean for that treatment group from one experiment (n = 4 experiments). *C–F*, Data collected from individual experiments performed with control C57BL/6 and ΔdblGATA mice that received PBS or negatively selected eosinophils. *C*, Larval burdens 17 dpi in diaphragms. Reduction in larval burden in ΔdblGATA PBS recipients compared with C57BL/6 controls is similar to values we have reported previously [7]. *D*, Area of larvae recovered from diaphragms 17 dpi. Bars represent means from 75–90 larvae pooled from diaphragms of 3–4 mice. *E*, Cytokines in Ag-stimulated cultures of CLN cells collected 17 dpi. F, Mean number of CD4+IL-4+ cells in diaphragms of mice 15 dpi. C–F, Each

data set was collected from two or three experiments with similar results. Values represent mean ± SD; n = 3–4 mice. Significant differences were determined by ANOVA and Tukey's test. *p , 0.05, **p , 0.001, ***p , 0.0001.

Discussion

Host adaptation is highly evolved among parasitic worms, and there is ample evidence that helminths manipulate the immune response in ways that prolong their survival in the host or promote their dispersal in the susceptible host population. The importance of obtaining a detailed and thorough understanding of the means by which nematodes interfere with immunity lies in the potential for such knowledge to inform the design of approaches to controlling infection and preventing disease. Our previously published findings revealed the potential for eosinophils to promote survival of *T. spiralis* in muscle. Specifically, we reported that when PHIL or ΔdblGATA mice are infected with *T. spiralis*, larvae are cleared from skeletal muscle by an iNOS-dependent mechanism [7]. We show in this study that neutrophils and macrophages produce iNOS at sites of infection. Although markers of M2 macrophages were dramatically upregulated in diaphragm tissue of both eosinophil-ablated and WT mice, eosinophil-ablated mice upregulated NOS2 on day 17 post infection and large numbers of iNOS + neutrophils and macrophages were present among infiltrating cells. Among the macrophage/ monocyte populations, Ly6C+CD11b+F4/ 80+ cells showed the highest levels of iNOS. This indicates that inflammatory monocytes recruited from blood, rather than tissue resident macrophages, were the primary sources of iNOS [20]. Similar numbers of neutrophils and Ly6C +CD11b+F4/80+ monocytes/macrophages were present in diaphragms of WT mice, suggesting

that the effect in eosinophil-ablated mice is not a defect in recruitment of such cells, but rather the result of cytokines that influence the induction of NOS2 expression.

Taken together, the results support a model in which eosinophils act locally, either directly or indirectly, to prevent the development of iNOS-producing neutrophils and macrophages capable of parasite killing. A direct effect of eosinophils on macrophage phenotype has been described recently. By virtue of their production of IL-4, eosinophils have been shown to promote alternative activation of macrophages in mouse adipose tissue, thereby promoting glucose tolerance and protecting against diet-induced obesity [21]. Furthermore, a recent report has shown that M2 macrophages proliferate in the pleural cavities of mice infected with *L. sigmodontis*, and that proliferation in situ is under the influence of IL-4 [22]. Our findings are not incompatible with local proliferation of M2 macrophages in muscles of *T. spiralis* infected mice, making the potential influence of eosinophils on local macrophage populations of considerable interest.

Previous work did not determine whether NO-dependent clearance of *T. spiralis* resulted from direct toxicity to larvae or from nitrosative damage to nurse cells [7]. Our results demonstrate that *T. spiralis* larvae are susceptible to direct killing by NO. Susceptibility is evident in newborns but increases during the period of rapid growth between 4 and 14 d after invasion of muscle cells [23]. These results suggest that NO can mediate killing of parasitic worms in response to infection in a natural host. Similarly, NO has been implicated in helminth killing in mice vaccinated against *S. mansoni* or colonized with *Brugia malayi* [24, 25]. NO produced by endothelial cells and macrophages kills *S. mansoni* larvae *in vitro* [26], and susceptibility is age dependent; however, in contrast with *T. spiralis*, older *S. mansoni l*arvae

show greater susceptibility [27]. In the *B. malayi* model, treatment of mice with an inhibitor of NO synthase abrogates resistance [24, 25]. Therefore, it is apparent that in contrast with the well-established role for Th2 immune responses in expulsion of intestinal worms, reactive nitrogen species produced during Th1 immune responses can be effective in host defense against tissue-dwelling parasitic worms.

We found that *T. spiralis* larvae became remarkably resistant to NO as they approached maturity. Antioxidant enzymes likely afford protection to the parasite [28], and expressed sequence tag analysis predicted that mature *T. spiralis* muscle larvae transcribe three types of antioxidant genes, specifically, thioredoxin peroxidase, peroxiredoxin, and glutathione peroxidase [29, 30]. These transcripts are less frequent in NBL, compatible with immature larvae being more vulnerable to oxidative or nitrosative damage [29–31] and consistent with results of our *in vitro* and *in vivo* experiments. Eosinophils appear to protect larvae during a window of susceptibility to oxidative and nitrosative stress.

Before destruction of larvae in eosinophil-deficient mice, nurse cell development was impaired and larval growth was inhibited. We have not yet determined whether this inhibition is the result of an immune response that develops in the absence of eosinophils or it reflects the parasite's dependence upon factors produced or induced by eosinophils. Parasite growth modulation by the immune system has been shown in other helminth infections. For example, T cells facilitate growth of *S. mansoni* by exerting non-cognate influence on MHC class II+ APCs [32]. In addition, development of the filarial nematode *L. sigmodontis* is transiently delayed in the absence of IL-5 or eosinophils, and *B. malayi* development improves in the presence of T cells and NK cells [33–35]. Although the role of lymphocytes may vary across

infections, the accumulating evidence supports a model in which innate immune cells influence the rate of growth and development of parasitic helminths.

We speculate that inhibition of larval growth would lengthen the period of susceptibility to NO-mediated killing and promote clearance of larvae. Consistent with this notion was the finding that larvae grew normally in IL-10–deficient mice, which demonstrate less dramatic parasite clearance but strong iNOS production [10]. The mechanism(s) behind compromise of larval growth in eosinophil-ablated mice remains to be elucidated. Angiogenesis is a prominent feature of nurse cell development, during which infected myotubes develop a surrounding vascular network that is presumed to support the parasite [36]. Eosinophils and M2 macrophages can promote angiogenesis [37–40]. If vascularization is compromised in the absence of eosinophils, both nurse cell differentiation and parasite growth may be inhibited, a hypothesis that we are currently testing.

T. spiralis can live for years in skeletal muscles of its host [41]. Prolonged survival requires that the worm suppress the host immune response or block its effects. Our data indicate that eosinophils directly or indirectly inhibit a Th1 immune response that induces the production of larvicidal NO; survival of the parasite is correlated with Th2 immunity. Eosinophils can promote Th2 responses by different mechanisms. It has been reported that human eosinophils expressing IDO catabolize tryptophan to KYN that subsequently causes apoptosis of Th1 cells [18]. This phenomenon has not been documented in murine eosinophils; however, we hypothesized that mouse eosinophils might have a similar influence, possibly by promoting IDO production in other cells. We evaluated the numbers of IDO-producing cells and IDO gene expression in diaphragms of WT, PHIL, and ΔdblGATA mice. In addition, we

measured KYN production by Ag-stimulated CLN cells or leukocytes isolated from infected diaphragms. None of these measures was affected by eosinophil deficiency. Mononuclear cells rather than granulocytes were the dominant sources of IDO in all strains. These results do not support an eosinophil- dependent role for IDO in inhibiting a Th1 response in WT mice.

We found that *T. spiralis* infection progressed normally in the absence of the MBP or EPO, indicating that these granular proteins do not contribute to parasite growth and survival, or to immune modulation. Another granular protein, human eosinophil- derived neurotoxin (an ortholog of the mouse eosinophil- associated RNase-2), has been shown to induce dendritic cell maturation and expansion of Th2 responses by virtue of its ability to activate TLR2 [42]. We did not detect a difference in dendritic cell numbers or maturation (as evidenced by MHC class II and CD86 expression) between WT and eosinophil-ablated mice in the CLN or diaphragm (data not shown). Our findings to date do not support a role for granular proteins in eosinophil-mediated regulation.

Basophils produce IL-4 and have the potential to influence immunity to *T. spiralis* [43]. Eosinophil ablation in ΔdblGATA mice was associated with a modest but significant reduction in the number of basophils in the SPLs of uninfected mice and infected mice at 2 dpi. It is not obvious that these differences would affect the immune response in skeletal muscle that develops 10 d later. Furthermore, improved growth and survival of larvae after transfer of eosinophils (between 5 and 15 dpi) reduces the likelihood that basophils have an important role in protecting larvae. Nevertheless, the data require further consideration of the potential influence of the basophil in larval survival and immune regulation.

Eosinophil transfer, during a limited but critical period of larval development, significantly improved growth and survival of larvae while simultaneously increasing Th2 cell migration to sites of infection. The results support the eosinophil as a regulator of local immunity. Despite the local effect, the transfer protocol did not alter the cytokine response of CLN cells in Ag re-stimulation assays. It is possible that the delivery schedule or number of cells transferred was insufficient to have an effect in regional lymphoid tissue, or that the assay is not sufficiently sensitive to detect those effects. In murine models of allergic lung disease, local Th2 cytokine production is reduced in eosinophil-ablated mice, a result that derives from reduced T cell recruitment into the lung [44, 45]. Similarly, mice deficient in the eosinophil chemotactic factors CCL11 (eotaxin-1) and CCL24 (eotaxin-2) show reduced cellular infiltrates and Th2 cytokine production in the lung [46], and supplementing ΔdblGATA mice with CCL11 enhances the Th2 response [44]. Adoptive transfer of eosinophils or eosinophils and CD4 T cells reconstitutes disease in ΔdblGATA and PHIL mice, respectively [44, 45]. Transfer of eosinophils deficient in IL-13 failed to restore disease, documenting IL-13 as a critical mediator of the regulatory effect of eosinophils in allergic airway disease [44, 47]. Our previous studies showed that STAT6-deficient mice do not clear muscle larvae, whereas IL-10-deficient mice upregulate NOS2 and clear parasites, suggesting that IL-10 may be more important than IL-4/IL-13 in protecting larvae against NO-mediated killing [10].

In addition to cytokines and granular proteins, eosinophils express MHC class II and present Ag, promoting Th2 differentiation in the context of helminth infection and allergic asthma [48, 49]. Eosinophils in *T. spiralis* infected mice upregulate surface expression of MHC class II and CD86 (V. Fabre and J.A. Appleton, unpublished observations), affording them the

potential to influence the development of T cell subsets via Ag presentation and constitutive expression of IL-4 [50].

Under homeostatic conditions, mouse eosinophils are present in the uterus, thymus, intestine, and mammary gland, where they have been associated with processes of cellular growth and differentiation [51]. The effect of eosinophils on macrophage phenotype in adipose tissue also occurs in the absence of infection [21]. Neither IL-5 deficiency nor overexpression of IL-5 impacts survival of *T. spiralis* muscle larvae [52–54], whereas ablation of the eosinophil lineage has a profound effect [7]. These contrasting results indicate that eosinophilia is not necessary for eosinophil-mediated immune regulation during muscle infection by *T. spiralis*. Thus, innate eosinophil functions, rather than those promoted by adaptive immune cells, are likely to be central in this context.

Parasite protection by eosinophils may benefit the host by preserving the antigenic stimulus for a Th2 response that prevents reinfection of the intestine. This limits the risk for overburdening the host, while at the same time reducing injury to skeletal muscle that is associated with parasite clearance. Dissection of the functional attributes of eosinophils, and identification of the cells with which they interact to exert their regulatory influence, will be crucial next steps in determining how these findings can be applied in developing new tools to prevent and control parasitic infections that continue to plague human and animal populations.

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Disclosures

The authors have no financial conflicts of interest.

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CHAPTER 3

Eosinophils and STAT6 regulate *Trichinella spiralis* muscle infection by controlling parasite growth

Abstract

The parasitic nematode *Trichinella spiralis* establishes chronic infection in the skeletal muscle. The muscle phase of infection is characterized by tissue and blood eosinophilia. Using two models of eosinophil-ablated mice, we have previously shown that larval growth and survival are significantly compromised in the absence of eosinophils and that this correlates with reduced Th2 immunity. We show here that reduced Th2 cell accumulation at infection sites is caused by impaired Th2 cell production in draining lymph nodes. Defective Th2 cell accumulation did not correlate with the expression pattern of chemokines that direct the migration/activation of T cells nor the ability of T cells to migrate into antigen-bearing tissue. Moreover, studies using STAT6-/- and IL-13-/- mice revealed that the IL-4/STAT6 axis regulated parasite growth. Impaired parasite growth in eosinophil-deficient mice correlated with increased expression of genes associated with nutrient deprivation (AMPK and INSR), but muscle or larval glycogen contents were not affected by eosinophil deficiency. These results implicate the immunoregulatory roles of eosinophils in acquired immunity and nutrient uptake during chronic nematode infection in which the eosinophil functions as a pivotal regulator of immunity.

Introduction

Muscle infection with *Trichinella spiralis* induces strong Th2 responses [1-3]. Blood and tissue eosinophilia is a characteristic feature of *T. spiralis* infection. Th2 cytokines IL-4 and IL-13 exert their biological effects via activation of the transcription factor signal transducer and activator of transcription protein 6 (STAT6) [4, 5]. STAT6 is also important in IL-4 induced commitment of CD4 T cells to the Th2 phenotype [4] and in the induction of

eosinophilia [6, 7]. Eosinophils are capable of affecting T cell activation directly or indirectly by secreting Th2-inducing cytokines such as IL-4 and IL-25 [8, 9], by secreting/driving the production of chemokines that attract T cells [10, 11], or by functioning as antigen presenting cells [12, 13].

During *T. spiralis* infection, Th2 immunity can develop in the absence of eosinophils but eosinophils promote the propagation of the Th2 response in the muscle [14, 15]. We have previously showed that Th2 cell accumulation at sites of infection is impaired in eosinophil-ablated mice, correlating with increased numbers of classically activated macrophages and inducible nitric oxide synthase (iNOS)+ neutrophils. Growing larvae were killed by nitric oxide (NO). The reduced Th2 immune response in the skeletal muscle was associated with impaired parasite growth. Restoring eosinophils to ablated mice improved Th2 cell recruitment and larval survival in the muscle.

The Th2 dominant immune response invoked by helminths modulates glucose homeostasis by regulating insulin action [16, 17]. Glucose absorption becomes maladaptive in the settings of a dysregulated immune response such as Th1 immunity causing type-2 diabetes [18, 19]. Immune cell and adipocyte-derived Th2 cytokines activate macrophages to an alternatively activated phenotype (M2) and inhibit insulin resistance in adipose tissue [16, 20]. In the context of helminth infection setting, chronic exposure to *Schistosoma mansoni* or parasite antigens prevented metabolic diseases such as diabetes mellitus [21, 22], and mice infected with *Nippostrongylus brasiliensis* were protected from diet induced obesity and insulin resistance [16]. In addition to this systemic effect, parasitic helminths derive nutrients from the host, and

anti-helminthic drugs such as albendazole work by blocking glucose absorption by parasites [23, 24].

Although skeletal muscle is the primary site for insulin-stimulated glucose utilization [25], very little is known about nutrient homeostasis in myotubes infected with the intracellular parasite *T. spiralis*. It has been reported that *T. spiralis* infection causes hypoglycemia and increase in muscle glycogen content [26]. Moreover, using C^{14} tagged glucose, it was shown that nurse cells that contain infective *T. spiralis* larva can import glucose from the host [27]. Addressing the potential effect of immunity on parasite nutrient uptake and the establishment of chronic infection is important in understanding how nutrient homeostasis is regulated in response to parasitism.

Since Th2 immune cells take residence in metabolic tissues, we investigated how these immune cells accumulate in the muscle and direct regulation of nutrient metabolism that may impact parasite growth. We also used STAT6-/- and IL-13-/- mice to further investigate the physiological relevance of Th2 immunity on parasite growth. Our findings indicated that eosinophils promote Th2 immunity in the skeletal muscle by enhancing Th2 cell proliferation in draining lymph nodes and genes related to nutrient starvation are increased in the muscle tissue of eosinophil-deficient mice. This observation implicated Th2 mediated immunity as the molecular basis for parasite growth and nutrient homeostasis during chronic infection by a parasitic nematode.

Materials and Methods

Rats and mice

Adult Albino Oxford (AO) strain rats were produced and maintained in the Baker Institute vivarium. STAT6-/- [4], PHIL [28], and ΔdblGATA [29] (C57BL/6 background) mice were bred at Cornell Transgenic Mouse Core Facility and progeny were transferred to the Baker Institute. PHIL mice were genotyped as described previously [28]. IL-13-/- mice were a gift from Dr. Avery August (Cornell) and Dr. Thomas Wynn (NIAID). $Arg1^{flox/flox}$;Tie2cre mice were a gift from Dr. Thomas Wynn (NIAID). $C57BL/6^{NHsd}$ mice (henceforth referred to as wild type [WT]) were purchased from Taconic. Animal care was in accordance with the guidelines of the American Association for Accreditation of Laboratory Animal Care and experiments were performed with the approval of the Institutional Animal Care and Use Committee of Cornell University.

Parasite and antigens

Trichinella spiralis first-stage larvae (L_1) and newborn larvae (NBL) were recovered from rats as described previously [30, 31]. For synchronous infection, 20,000 NBL suspended in serum-free DMEM (Mediatech, Inc.) were delivered by retro-orbital injection. For oral infection, L_1 were suspended in 2% nutrient broth (Difco)- 0.6% gelatin (Fisher Scientific) and doses of 300 L_1 were administered by gavage. When immune responses elicited by the two routes of infection were compared, oral infections were started four days prior to the intravenous infections, as *T. spiralis* does not mature to adult worms and produce parasitic NBL until 4 days after establishment in the intestine [32]. Mice were euthanized by CO_2 inhalation at the times

indicated in each experiment. Muscle larvae burdens were assessed in whole carcasses as described previously [14]. Soluble extracts of L_1 were prepared as described previously [33].

Bone Marrow chimeras

Wild-type (WT) C57BL/6 or STAT6-/- recipient mice were exposed to two doses of 550 rad total body irradiation in 3hr intervals. The following day, irradiated mice were injected with 2–5 x 10^6 bone marrow cells from WT mice. Recipient mice were left for 8–10 wk to reconstitute, and were given water supplemented with 2 mg/mL neomycin sulfate (Bio- Shop) for the first 2 wk. Hematopoietic reconstitution of chimeras was confirmed by flow cytometry.

Flow cytometry

Cells were recovered from individual diaphragms and cervical lymph nodes (CLN) as described [1]. Cells were surface stained as described [15] with PE-Cy7conjugated anti-CD4, PE conjugated anti-CD44, and FITC conjugated anti-CD62L. Reagents were purchased from eBioscience. Cells were analyzed on a FACSCalibur with CellQuest software (Becton, Dickinson and Company).

BrdU labeling

For 5-bromo-2'-deoxyuridine (BrdU) labeling experiments, BrdU was administered to mice by intraperitoneal injection (1 mg per mouse) and CLN were removed 24 h later and recovered cells were cultured *ex-vivo* for 6 h with 250 ng/ml ionomycin (Sigma-Aldrich), 50 ng/ml PMA (Sigma-Aldrich), and 1 µg/ml Brefeldin A (BD Pharmingen). Cells were surface stained with PE conjugated anti-CD4 before they were fixed and permeabilized for

intracellular IL-4 (APC conjugated anti-IL-4) and BrdU detection by flow cytometry according to the manufacturer's protocol (FITC BrdU Flow Kit-BD Pharmingen).

T cell isolation and adoptive transfers

CD4+ T cells were isolated from CLN of infected WT and ΔdblGATA donor mice (14dpi) and enriched by magnetic negative selection using CD4 T Cell Isolation Kit II (Miltenyi Biotec). Average purity was 93%. Cells were labeled with the intracellular fluorescent dye carboxyfluorescein succinimidyl ester (CFSE) (Invitrogen) according to the manufacturer's protocol. 3-5 x 10^6 CD4 T cells suspended in sterile 200 µl of PBS were delivered by retro-orbital i.v. injection at 14dpi. Twenty-four hours after adoptive transfer, leukocytes were recovered from diaphragms and CLN of recipient and control mice, and T cell activation markers ($CD44^{hi}$ $CD62L^{lo)}$ were evaluated on CFSE tagged CD4+ T cells using flow cytometry.

Immunohistochemistry

Immunohistochemistry was conducted as described previously [34]. Phosphorylated STAT-6 was stained with rabbit polyclonal anti-p-STAT6 (Abcam) and sections were counterstained with hematoxylin (Fisher). Microscopy was performed under 20X magnification using a BX51 microscope (Olympus).

Relative Expression of mRNA Transcripts by qRT-PCR

Total RNA was isolated from diaphragm tissue using the TRIZOL reagent (Invitrogen, Carlsbad, CA) and cDNA was prepared using the SuperScript III First-Strand cDNA Synthesis System (Invitrogen). Pre-designed TaqMan® gene expression assay primers and probes for AMP-activated protein kinase, alpha 1 (AMPK - Mm01296700_m1), Insulin Receptor

(INSR - Mm01211875_m1), IL-13 (Mm00434204_m1), CCL11 (Mm00441238_m1), CCL22 (Mm00436439_m1), CCL17 (Mm01244826_g1), CXCL7 (Mm00470163), CCL20 (Mm01268754), LIF (Mm00434762_g1), Cmtm2a (Mm00459052_m1) and CCR1L1 (Mm00432606_s1) were purchased from Applied Biosystems. Quantitative RT-PCR (qRT-PCR) experiments were performed as described [15].

PCR array

We performed PCR based array on pooled cDNA samples (n = 3 per pooled sample), using the Mouse Chemokines and Receptors RT-PCR Array (SABiosciences) and the Mouse Angiogenesis RT-PCR Array (SAbiosciences). The arrays were performed and analyzed according to the manufacturer's recommendations; the maximum Ct value was set at 40. Fold differences were calculated using the ΔΔCt method.

Statistical analysis

All experiments were repeated two to four times with similar results, except for the chimera study which was done once. Means ± SD were calculated from data collected from individual mice. Significant differences were determined using Student's *t* test or ANOVA with Tukey's post-hoc test for multiple means (GraphPad Prism 5 software).

Muscle glycogen assay

Muscle glycogen content was measured by phenol-sulfuric acid colorimetric assay [35] in mouse masseter muscles, or first-stage larvae (L_1) isolated from whole carcasses of infected mice [14].

NO fluorometric assay

Total NO end products (nitrates and nitrites) were measured in CLN culture supernatants as described [14].

Cytokine ELISA

Cells from CLNs were obtained and cultured and IL-4, IL-5, IL-10, IL-13, and IFN-γ were assayed in culture supernatants by ELISA as described previously [14].

Results

Eosinophils are not necessary for chemokine expression in the muscle

T cell accumulation in lung tissue induced in an allergic disease model was dependent on chemokines that were up-regulated by eosinophil derived IL-13 [36]. In our parasite infection model, masseter muscle was evaluated for local chemokine production using a Chemokine and Receptors PCR Array (Qiagen) (Appendix B-1), and results were validated by qRT-PCR (Appendix B-2). Chemokine production in eosinophil-deficient mice was not defective. In fact, there was overproduction of CCL11, CCL22, IL-13 and CCL17 (Fig. 3.1A). Overproduction of chemokines at sites of infection may have resulted from dysregulated T cell trafficking in eosinophil-deficient mice. Extravasation of effector T cells in inflammation also requires proper T cell priming in draining lymph nodes and subsequent adhesion of T cells to endothelium. We measured T cell priming and adhesiveness by evaluating cell surface expression of CD69 and CD18 on T cells respectively. WT and eosinophil deficient mice had comparable populations of CD4+ CD69+ and CD8+CD69+ T cells in their CLN and diaphragm

(Fig. 3.1C). The surface density of CD69 on existing CD4 and CD8 T cells was also not affected by eosinophil deficiency (Fig. 3.1D). WT and eosinophil-deficient mice also had similar percentages of CD18+CD4+ cells in the CLN (Fig. 3.1B). Taken together, the results suggested that eosinophils do not have a role in determining chemokine and integrin expression or T cell priming.

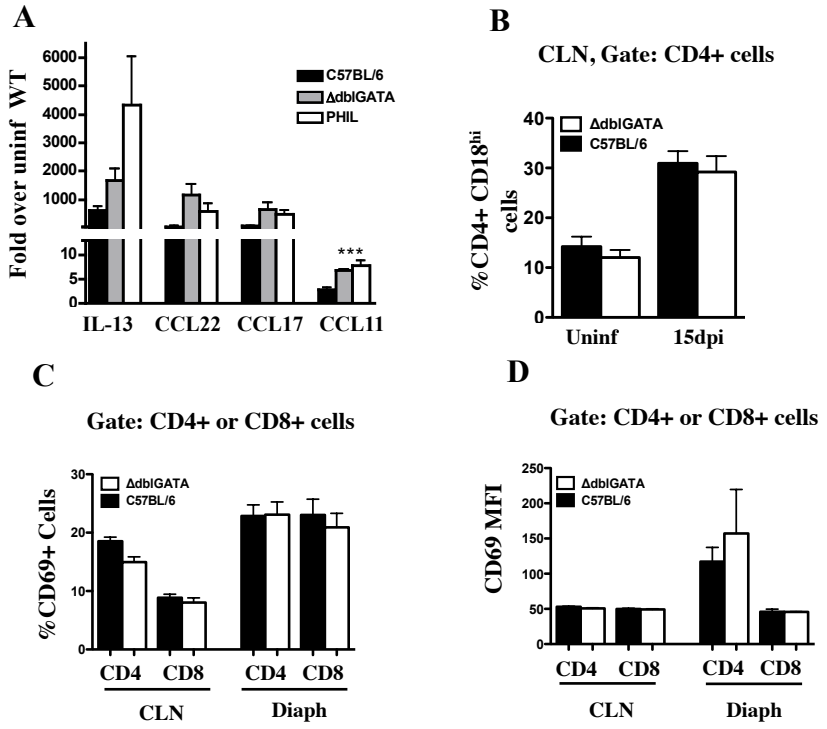

FIGURE 3.1. Chemokine expression in the muscle.

A, Quantitative RT-PCR analysis of IL-13, CCL11, CCL17 and CCL22 in masseter muscles of PHIL, ΔdblGATA and WT mice, 14 days post infection (dpi). *B,* Percentage of CD4+CD18+ cells recovered from CLN of ΔdblGATA and WT mice, 14 dpi. *C,* Percentage of CD69+ CD4+ and CD69+ CD8+ cells and *(D)* mean fluorescence intensity (MFI) for CD69 among CD4+ and CD8+ cells recovered from CLN and diaphragm of ΔdblGATA and WT mice, 14 dpi.

Values represent means ± SD, n = 3 - 4 mice. ***, $p < 0.0001$.

T cells migrate efficiently in eosinophil-deficient mice

Trans-endothelial migration of primed T cells to sites of infection or injury is dependent on the ability of both T cells and endothelial cells to express complementary adhesion molecules. We hypothesized that T cells may not properly infiltrate infection sites in eosinophil-deficient mice if endothelial cells and/or T cells did not display proper adhesion molecules. Although T cell CD18 expression was not altered in the absence of eosinophils, other integrins can modulate T cell recruitment in inflammation. To test this hypothesis, we performed adoptive transfers of CFSE labeled WT CD4+ T cells from *T. spiralis*-infected mice (14dpi) to *T. spiralis*-infected eosinophil-deficient and WT recipients (14dpi) and evaluated the tissue distribution of labeled cells. Briefly, 5 x 10^6 CFSE labeled CD4 cells collected from donor mice at 14dpi were transferred and migration was evaluated 24 h later (Fig. 3.2A).

Because meaningful numbers of transferred T cells at infection sites were not detected in WT recipients that served as positive controls (most likely because cells disseminate in whole body skeletal muscle), an alternative strategy was used to measure extravasation of adoptively transferred cells by evaluating $CD44^{hi}CD62L^{lo}$ cell surface marker expression in the CLN of recipients after 24 h. Flow cytometry analysis revealed that the majority of transferred CD4+ T cells from infected mice (>75%) displayed the $CD44^{hi}CD62L^{lo}$ phenotype suggesting that these cells egressed from infection sites (Fig. 3.2C). In addition, transferred T cells were fully functional because leukocyte recruitment at sites of infection improved in eosinophil ablated CD4+ T cell recipients (Fig. 3.2B). CD4+ T cell transfer from ΔdblGATA donor mice yielded similar results (Fig. 3.2D- F). Collectively, these results indicated that CD4+ T cells

produced in *T. spiralis*-infected eosinophil-deficient and WT mice have a similar phenotypic and homing property, and that endothelial cells in eosinophil-ablated mice can facilitate transmigration of CD4+ T cells to infection sites.

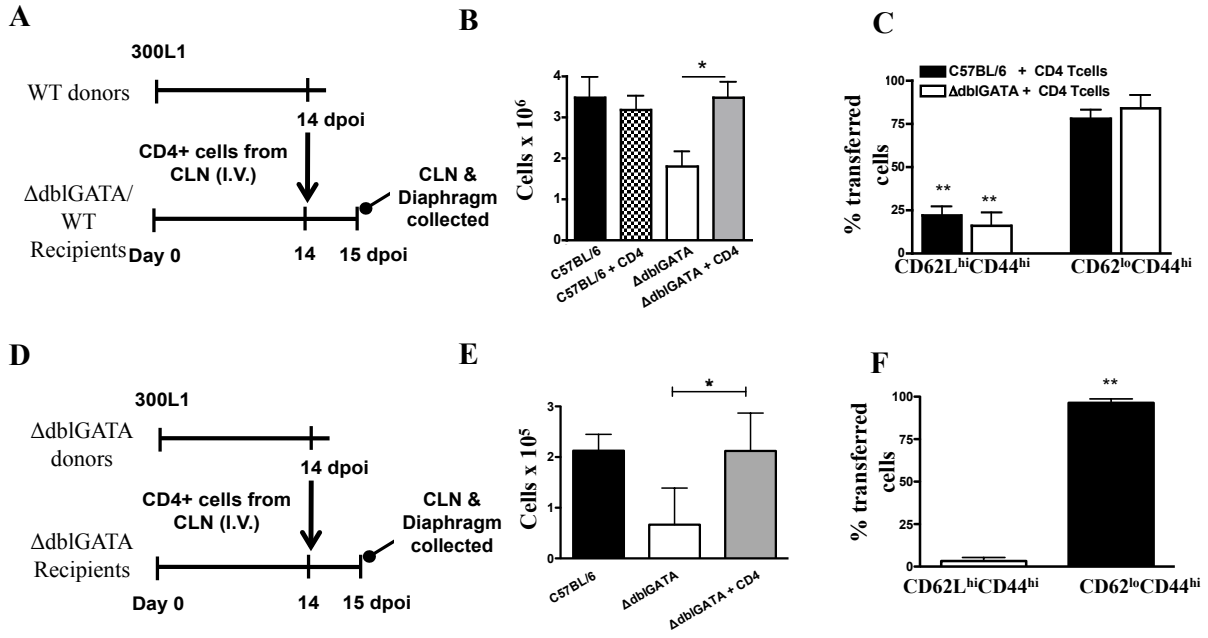

FIGURE 3.2. T cell trafficking in eosinophil-deficient mice.

To perform T cell migration studies, CFSE-labeled CD4+ T cells from *T. spiralis*-infected (*A*) or ΔdblGATA (*D*) mice were transferred into *T. spiralis*-infected recipients (5 x 10^6 cells/mouse). CLN and diaphragms were harvested from T cell recipients and controls after 24 h. CD44 and CD62L expression on transferred WT (*C)* or ΔdblGATA (*F)* CFSE+ T cell populations were evaluated in the CLN. Total muscle leukocyte counts were evaluated in diaphragms of mice that received T cells from WT (*B*) or ΔdblGATA (*E*) donors (4% of total leukocytes in ΔdblGATA T-

cell recipients were CD4+ cells). *Values represent means ± SD, n = 3 - 4 mice.* ∗, $p < 0.05$; ∗∗, $p < 0.001$.

Th2 cell expansion is compromised in the absence of eosinophils

The apparent reduction in local Th2 cell populations in eosinophil-deficient mice may be caused by a defect in proliferation of Th2 cells. Proliferation in WT, PHIL and ΔdblGATA mice was measured at 10, 13, and 15 dpi in the CLN, spleen (SPL) and mesenteric lymph nodes (MLN). BrdU labeling in CD4+ or IL-4+CD4+ was equivalent in the MLN or SPL of all mouse strains tested (Appendix C-1). Homeostatic proliferation of naïve CD4+ T cells was similar between WT and eosinophil-ablated mice (Fig. 3.3A, B). In contrast, at 13dpi the percentage of BrdU labeled IL-4+CD4+ cells in CLN was significantly higher in WT mice compared to PHIL mice, suggesting that Th2 cell proliferation is compromised in the absence of eosinophils (Fig. 3.3C). A similar trend was evident in two experiments using ΔdblGATA mice with a similar design but the results did not achieve statistical significance. This was consistent with the overall stronger phenotype exhibited in *T. spiralis*-infected PHIL mice compared to ΔdblGATA mice [14]. Thus, the decreased Th2 cellularity we observe in tissues of eosinophil-deficient mice was attributed in part to reduced production of cells in draining lymph nodes.

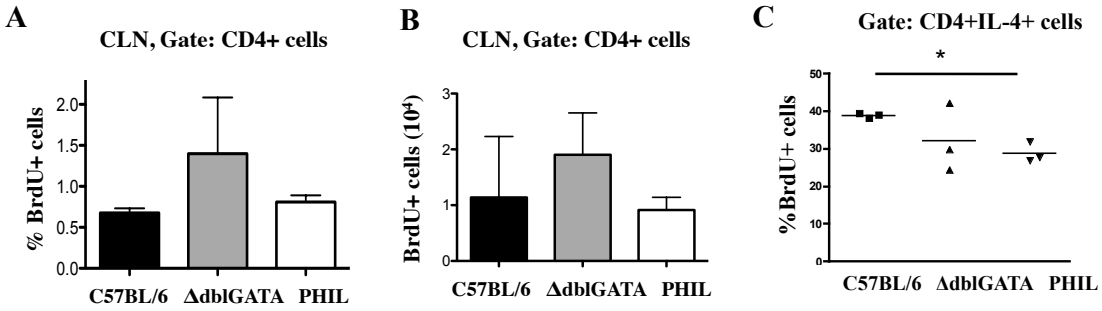

FIGURE 3.3. Proliferating T cells in draining lymph nodes.

To evaluate CD4+ T cell proliferation, *T. spiralis*-infected and uninfected WT, ΔdblGATA, and PHIL mice were given BrdU (1 mg/mouse i.p.) and CLN cells were harvested the following day. Proliferative capacity of CD4+ T cells in uninfected eosinophil-ablated and WT mice *(A, B).* CD4+IL-4+ cell proliferation, 13 dpi *(C). Values represent means ± SD, n = 3 - 4 mice.* ∗, $p < 0.05$.

Requirement for STAT6 for parasite growth but not survival

The reduced frequency of Th2 cells in *T. spiralis*-infected muscle was associated with impaired parasite growth and parasite death. To further investigate the role of Th2 cells in parasite development, larval growth and survival was evaluated in synchronously infected STAT6-/- and IL-13-/- mice. Larva development progressed normally in IL-13-/- mice but not in STAT6-/- mice, suggesting that IL-4 promotes parasite growth through STAT6 signaling (Fig. 3.4A, B). While parasite growth was compromised by STAT6 deficiency, parasite survival was not affected (Fig. 3.4E). Immunostaining for p-STAT6 in uninfected and *T. spiralis* -infected WT tongue revealed that both skeletal muscle cells and hematopoietic cells signal via STAT6 (Fig. 3.4C).

Bone marrow chimeras were developed to determine if STAT6 competent hematopoietic cells were sufficient to promote parasite growth. WT and STAT6-/- mice were irradiated and reconstituted with WT bone marrow (BM reconstitution was 100% successful) , and parasite growth was compared between *T. spiralis* -infected chimeric and control mice.

Parasite growth improved in STAT6-/- mice that were reconstituted with WT bone marrow, suggesting that STAT6 competent hematopoietic cells contribute to improved parasite growth (Fig. 3.4D). Differences in parasite measurement between chimeric and control STAT6-/- mice did not achieve statistical significance, suggesting that hematopoietic cells may need to work collaboratively with skeletal muscle cells to support parasite growth. Although IL-13 did not impact larval growth in the muscle of orally infected mice, IL-13 deficiency caused delayed worm expulsion from the intestine, consequently causing a moderate increase in muscle larval burden (Fig. 3.4F, G). These results demonstrated that the IL-4/STAT6 signaling axis regulated parasite growth but not survival in the muscle.

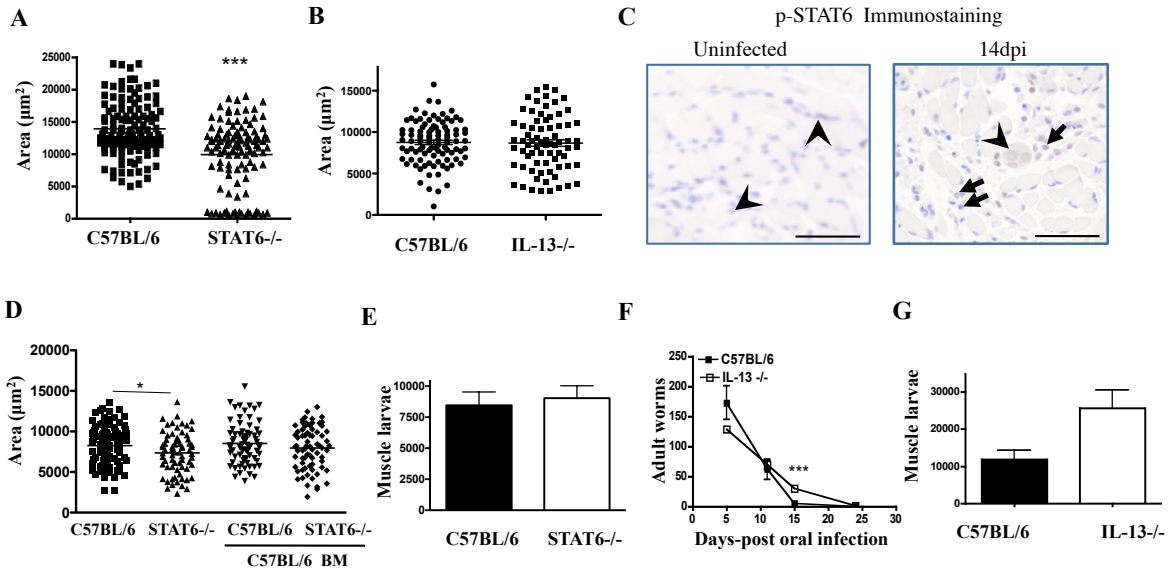

FIGURE 3.4. STAT6 signaling in hematopoietic cells is essential for parasite growth.

Estimated area of larvae recovered from synchronously infected STAT6-/- and WT mice, 17dpi (*A*); IL-13-/- and WT mice, 17dpi (*B*). Cross section of infected tongue stained with p-STAT6 (scale bars = 20 µm). *C*, p-STAT6 staining in skeletal muscle nuclei (arrowheads) and

eosinophils (arrows). Bone marrow cells from WT mice were used to reconstitute STAT6-/- and WT recipients as described in *Materials and Methods* and area of larvae recovered from recipients was measured, 17dpi (*D*). Larval burdens in muscles of synchronously infected STAT6-/- and WT mice, 24dpi (*E*). Kinetics of adult worm expulsion from small intestines of orally infected WT or IL-13-/- mice (*F*). Larval burdens in muscles of orally infected WT or IL-13KO mice, 28 dpi (*G*). *Values represent means ± SD, n = 3 - 4 mice.* ∗, $p < 0.05$; ∗∗∗, $p < 0.0001$.

AMPK and INSR are upregulated in muscles of eosinophil-ablated but not STAT6-/- mice

To assess the influence of eosinophil deficiency on muscle nutrient homeostasis, transcription of genes associated with nutrient starvation (AMPK and INSR) were measured in the skeletal muscles of infected ΔdblGATA, PHIL, STAT6-/- and control mice. AMPK and INSR transcription levels were significantly increased in muscles of infected eosinophil-deficient mice compared to infected WT mice. (Fig. 3.5A, B). In contrast to these findings, skeletal muscle glycogen levels were similar in tissues of infected eosinophil-deficient and WT controls (Fig. 3.5D). Larvae harvested from these mice also had comparable glycogen content (Fig. 3.5E). However, muscle glycogen content increased after infection (Fig. 3.5E), consistent with previous findings that show that glucose transport is enhanced in infected myotubes (27, 37). These results suggest that eosinophils may impact muscle larvae growth by regulating nutrient homeostasis in infected muscle but do not impact whole muscle glycogen synthesis and, thereby, glucose uptake. AMPK and INSR gene expressions did not increase in STAT6-/- mice (Fig.

3.5C), suggesting that mechanisms of parasite growth modulation may be different in eosinophil-deficient versus STAT6-/- mice.

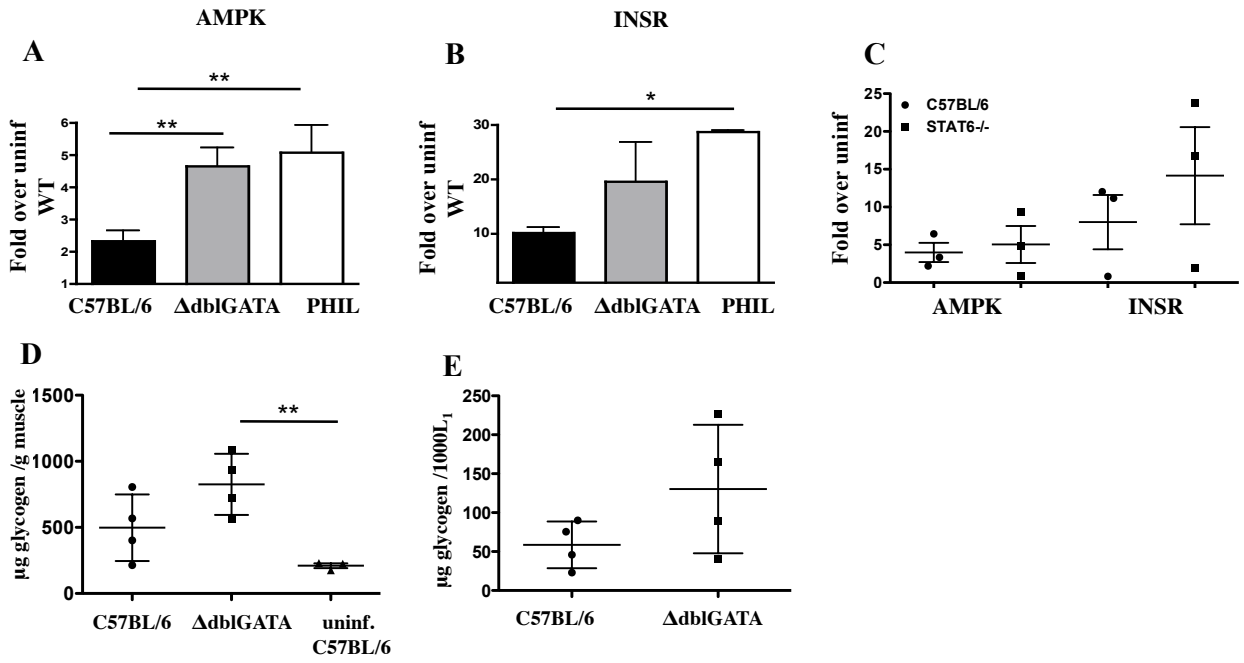

FIGURE 3.5. Examination of glucose homeostasis in the muscle.

Quantitative RT-PCR analysis of AMPK and INSR in masseter muscles of PHIL, ΔdblGATA and WT mice (*A, B*) and STAT6-/- mice *(C, dots represent individual experiments)* at 17 dpi. Dots in (*C*) represent individual experiments. Glycogen concentrations in the masseter muscles of uninfected and infected male mice (26 dpi) (*D*) Glycogen measurement in *T. spiralis* L_1 isolated from infected ΔdblGATA and WT mice (26 dpi) (*E*). *Values represent means ± SD, n = 3 - 4 mice.* ∗, $p < 0.05$; ∗∗, $p < 0.001$.

Discussion

The eosinophil-T cell interaction paradigm has significance in eosinophilic diseases where Th2 inflammation is a common factor, such as asthma, cancer, gastrointestinal diseases, organ transplant rejection, as well as helminthic infection [15, 38-41]. In eosinophil-deficient mice, Th2 cell accumulation was reduced in muscle sites of *T. spiralis* infection. To determine the causes of reduced Th2 cell accumulation T cell chemokine expression, ability of T cells to traffic to infection sites, and proliferation of T cells in *T. spiralis*-infected WT and eosinophil-deficient mice were evaluated. We found that eosinophil deficiency has no negative impact on T cell recruitment or trafficking to infection sites. However CD4+IL-4+ T cell proliferation in draining cervical lymph nodes was reduced by 25% in the absence of eosinophils. T cell proliferation at sites of infection (data not shown) or in non-draining lymph nodes and spleen (Appendix C-1) was similar between WT and eosinophil-deficient mice. To ensure that T cells were activated normally, CD69 activation marker expression on T cells was evaluated. The expression of this molecule on CD4+ T cells from CLN, SPL and diaphragm of infected WT and ΔdblGATA mice was similar, suggesting that T cells are efficiently activated in eosinophil-deficient mice.

These results suggest that eosinophils influence production of effector Th2 cells in lymphoid organs and eosinophils are not necessary for localization of primed T cells to non-lymphoid antigenic sites. The mechanism by which eosinophils promote proliferation of Th2 cells is unclear. TCR engagement by eosinophils and/or IL-4-mediated signals promote the priming and proliferation of Th2 cells [9, 38, 42, 43]. Alternatively, eosinophils could affect other antigen presenting cells such as dendritic cells (DCs) that deliver Th2 proliferation signals

(such as IL-4) in conjunction with TCR engagement [44-46]. There are various mechanisms by which eosinophils have been shown to impact DC-T cell interaction. The human eosinophil granule protein (eosinophil derived neurotoxin), engages DC TLR2 that causes DC maturation and expansion of Th2 populations [44]. Other studies have shown that eosinophils can make DC-modulating cytokine IL-25 that augments Th2 responses [8], or inhibit Th1/Th17 responses via undetermined mechanisms [47, 48], that would indirectly enhance Th2 responses. Another study has shown that eosinophils induce the accumulation of myeloid DCs in draining lymph nodes and, in turn, Ag-specific Th2 effector cell production [46]. Thus, eosinophils can potentially regulate Th2 immunity via diverse mechanisms during *T. spiralis* infection.

We have previously shown that reduced numbers of Th2 cells are associated with impaired larval growth in eosinophil deficient mice [15]. Our results using STAT6-/- and IL-13-/- mice suggested that the IL-4/STAT6 arm of the immune system plays an important role in parasite growth. Although both infected muscle cells and inflammatory cells exhibited nuclear p-STAT6 staining, chimera studies showed that STAT6+/+ hematopoietic cells are sufficient to improve parasite growth in STAT6-/- mice. However, muscle larvae survival was not affected in intravenously infected STAT6-/- mice, most likely due to IL-10-mediated suppression of NO response that is required for clearing infection [1]. Larval burden was increased in muscles of orally infected IL-13-/- mice consistent with delayed worm expulsion, suggesting that this cytokine is critical for resistance to intestinal infection, but does not have a role during the muscle stage. These results are in keeping with the capacity of Th2 cytokines to promote intestinal worm expulsion by enhancing goblet and mast cell hyperplasia and intestinal muscle hypercontractility [49].

The cause for impaired parasite growth is not clear. We have previously tested the role of reactive nitrogen species on parasite growth using IL-10-/- mice, which develop a significant NO response [1, 15]. Although NO contributed to parasite death [1, 15], our results did not implicate NO in parasite growth retardation. Given the association between Th2 immunity and parasite growth, we hypothesized that reduced Th2 immune responses impair parasite growth by inducing mild insulin resistance in the skeletal muscle, which limits glucose availability to growing muscle larvae. Several studies have shown that Th2 cytokines produced by adipocytes and/or immune cells improved glucose tolerance both in the state of homeostasis and in the context of infection [16, 50]. For instance, STAT6-/- mice are resistant to diet-induced obesity [51], and exogenous IL-4 improves glucose homeostasis in obese WT mice [51]. Consistent with these findings, major metabolic tissue such as human skeletal muscle cells, liver and adipose tissues have been shown to be capable of responding via the IL-4/STAT6 signaling pathway under homeostatic conditions or in response to stimuli such as physical exercise [51, 52].

We performed a qRT-PCR analysis of genes associated with nutrient deprivation in infected muscles of eosinophil-deficient, STAT6-/-, and control mice. Our results showed that AMPK and INSR gene expressions were increased in muscles of infected eosinophil-deficient mice. AMPK is an important nutrient sensor that is activated by energy starvation (an increase in intracellular AMP: ATP ratio) [53-55]. An increase in AMPK gene expression coupled with increased INSR expression strongly suggested that glucose uptake was impaired in the absence of eosinophils. However, both muscle and parasite glycogen contents were similar between WT and eosinophil-ablated mice. Consistent with these findings, transcription of glycogen synthase

kinase 3 beta (Gsk3b), which negatively regulates glycogen synthesis, was not altered in the absence of eosinophils (Appendix C-2). The results do not completely exclude a role for variation in glucose uptake capacity on parasite development. Particularly, if glucose uptake is selectively compromised in infected cells, total muscle glycogen measurements may not be sensitive enough to detect differences. In addition, if larval glucose uptake is compromised at an early stage in the development of parasites, mature larvae glycogen measurements may not be particularly informative. An age-dependent metabolic analysis of muscle larvae or infected cells is required to rule out glucose starvation in eosinophil-dependent modulation of parasite growth. Moreover, since activation of AMPK/Gsk3b can be regulated after transcription, enzyme activity studies are crucial to further validate PCR results. Future studies must also consider the role of AMPK activity on other nutrient deficiencies (for e.g. amino acids).

Transcription of AMPK or INSR was not increased in muscles of STAT6-/- mice, suggesting that independent/ additional mechanisms of growth impairment are at play. Alternatively, this may also mean that the AMPK /INSR pathway does not regulate parasite growth. Most likely, different populations of immune cells colonize the skeletal muscles of STAT6-/- and eosinophil-ablated mice during *T. spiralis* infection. It is possible that the differences we observe in AMPK and INSR gene expressions in eosinophil deficient versus STAT6-/- mice is caused by a shift in metabolic requirements of infiltrating cells. Muscle or larval metabolic studies that exclude contaminating cells are essential to eliminate this variable.

Parasite growth may also be impaired if other aspects of nurse cell transformation are altered. *Trichinella spiralis* is an intracellular parasite that causes several morphological and biochemical changes in the infected cell, in order to create a niche that fulfills its needs. For

instance, parasitized myotubes are surrounded by discrete circumferential vascular networks and a thick collagen capsule that presumably supply nutrients and support to the enclosed larva. Pro-angiogenic Th2 leukocytes such as eosinophils and M2 macrophages may promote parasite growth by supporting local vascularization. For example, there is strong evidence that M2-like macrophages are involved in tumor angiogenesis by releasing pro-angiogenic growth factors such as IL-8, VEGF and MMP9 [56, 57]. A preliminary qRT-PCR based array study showed that transcription of pro-angiogenic genes such as fibroblast growth factor, hepatocyte growth factor, placental growth factor, matrix metalloproteinase 9, and angiopoetin 2 are equally increased in muscles of WT and PHIL mice (Appendix-A). However, eosinophil-ablation selectively increased transcription of anti-angiogenic genes (plasminogen, collagen XVIII and granulocyte-colony stimulating factor -Appendix-A), implicating eosinophil driven immune responses in promoting angiogenesis during muscle infection with *T. spiralis*.

Eosinophil containing infiltrates and STAT6 may also be contributing to the net deposition of collagen and other extracellular matrix components that support the nurse cell structure and integrity [58]. Eosinophils are capable of making important fibrogenic cytokines, such as TGF-β, IL-4 and IL-13 [59-61]. M2 macrophages produce Arginase-1 (Arg1) in a STAT6 dependent manner and Arg1 activity provides L-proline from arginine, an essential substrate for collagen synthesis [62]. We evaluated the role of macrophage Arg1 in parasite establishment using $Arg1^{flox/flox}$;Tie2cre mice that lack Arg1 in hematopoietic cells. Although Arg1 deletion in these mice is hematopoietic cell specific, infected skeletal muscle cells did not produce Arg-1 (Appendix C-7). Our results did not support a role for Arg-1 mediated collagen deposition on parasite growth (Appendix C-7). However, muscle burden was reduced in muscles of Arg1

deficient mice and this correlated with enhanced reactive nitrogen species in antigen-stimulated cultures of CLN cells (Appendix C-7). This was not surprising since Arg1 regulates iNOS activity, as both enzymes utilize a common substrate (l-arginine) [62].

Taken together, our results suggested that macrophage-specific Arg1 promotes parasite survival, most likely by limiting larvicidal inflammation, but does not impact parasite growth. These results do not exclude a role for collagen deposition in parasite growth since collagen synthesis can be regulated by other mechanisms including Arginase-2, which is abundant in hematopoietic and skeletal muscle cells [63, 64]. Moreover, $Arg1^{flox/flox}$;LysMcre mice infected with *Schistosoma mansoni* showed no impairment in hepatic fibrosis, suggesting that other mechanisms of collagen synthesis are at play during parasitic infections [65]. Parasite development may be inhibited if nurse cells become less hospitable due to compromised vascularization or collagen deposition.

In conclusion, our results indicated that a dysregulated Th2 immune response in *T. spiralis*-infected eosinophil-deficient mice impaired nutrient uptake which has an effect on parasite growth. We also demonstrated that growth of muscle larvae requires host STAT6 signaling in both hematopoeitic and skeletal muscle cells, but that a key product of STAT6 dependent signaling, Arg-1 is not required for the mechanism of STAT6 dependent growth. Understanding how host immunity impacts parasite metabolism might serve as an important therapeutic target to parasitic infections.

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CHAPTER 4

Summary and Conclusions

We have previously showed that in the absence of eosinophils, *T. spiralis* muscle larvae die in large numbers and local nitric oxide production was associated with parasite death [1]. We show here that eosinophil-deficient mice traffic fewer leukocytes to sites of infection, with a significant decrease in numbers of $CD4^+$ $IL\text{-}4^+$ and $CD8^+$ $IL\text{-}4^+$ T cells. This microenvironment moderately shifted macrophage polarization from alternatively activated (M2) to classically activated (M1) state and increased NO production by neutrophils. This shift in immune response correlated with impaired parasite growth before larvae were killed in the muscle. Eosinophil reconstitution significantly improved parasite growth and survival, and correlated with improved Th2 cell accumulation at infection sites. In contrast, infection progressed normally in mice that lack the eosinophil granule proteins, eosinophil peroxidase and major basic protein 1.

We hypothesized that eosinophils modulate T cell response by expressing Indoleamine 2,3-dioxygenase (IDO), which catabolizes tryptophan to kynurenine (KYN) that subsequently causes apoptosis of Th1 cells [2]. However, we did not find evidence that IDO activity via the kynurenine pathway of tryptophan degradation was affected by eosinophil deficiency. We also investigated the roles of chemokines in dictating Th2 cell localization. The results indicated no defect in recruitment of T cells to infection sites in eosinophil-deficient mice. On the contrary, T cells produced in *T. spiralis*-infected eosinophil-deficient mice displayed similar phenotypic and homing property as WT T cells, suggesting that eosinophil deficiency did not impact T cell priming or trafficking during *T. spiralis* muscle infection. However, Th2 cell proliferation in draining lymph nodes was moderately reduced in eosinophil-ablated mice,

implicating eosinophils in the amplification of Th2 cells. Eosinophils can potentially enhance the proliferative ability of Th2 cells by presenting antigen or producing cytokines that promote Th2 cell expansion [3-5]. Eosinophils may also influence Th2 immunity by regulating response of non-T cells. Basophils and mast cells promote Th2 responses during helminthic infections [6-8]. We observed a modest but significant reduction in splenic basiophilia in uninfected and infected (2dpi) ΔdblGATA mice. Although this suggested that eosinophils promote basophilia, it is most likely a redundant role since basophil numbers in ΔdblGATA mice quickly caught up to WT levels shortly after infection (5dpi). Nevertheless, the basophil effects on immune regulation and parasite survival merit further investigation. Studies using mast cell deficient W/wv mice [9] did not show a meaningful impact on cytokine response or muscle burden (Appendix C-5). However, because mast cells have a documented impact on intestinal worm expulsion [10], these experiments need to be replicated by i.v. injection of new born larvae (NBL) to achieve synchronous muscle infections.

Eosinophils may also directly modulate macrophage function via IL-4/IL-13 activation or by influencing dendritic cell (DC) maturation and/or activation [11-14]. The importance of DCs in various Th2 diseases has been evaluated using transgenic mice in which DCs or their function is conditionally deleted. In allergy asthma models, deletion of DCs caused failure in the development of Th2 immunity and eosinophilia [15, 16]. DC deletion also impaired Th2 effector responses, during infections with the parasitic helminths *Schistosoma mansoni* and *Heligmosomoides polygyrus* [17, 18]. Future studies will determine what aspect of the eosinophil directs Th2 cell expansion *in vivo* during *T. spiralis* infection. Figure 4.1a below represents a summary of mechanisms of immune regulation by eosinophils.

Reduced Th2 immune response correlated with impaired parasite growth. We further tested the role of Th2 immunity on parasite growth using STAT6-/- and IL-13-/- mice. STAT6-deficient mice cannot develop adaptive Th2 immune responses [19-21]. Although the role for STAT6 in regulating the murine immune response to *T. spiralis* intestinal infection is very well characterized [22], its role during muscle infection has not been extensively studied. Parasite survival in muscle is not affected in STAT6-/- mice synchronously infected by NBL injection, and this correlates with uninhibited IL-10 levels that potentially limit NO response [23]. STAT6-/- mice otherwise resembled eosinophil-deficient mice in terms of Th2 response reduction and parasite growth rate. Reconstitution with STAT6+/+ bone marrow improved parasite growth in STAT6-/- mice, although not to the level of growth rate evident in WT mice, suggesting that STAT6 activation in both hematopoietic and skeletal muscle cells contributes to parasite growth. By contrast, parasites grew at a normal rate in IL-13-/- mice. These results indicated that the IL-4 /STAT6 signaling axis regulates parasite growth, although both IL-4 and IL-13 can signal through STAT6 and have over-lapping functions.

Developing muscle larvae are known to inhibit immune-mediated destruction by stimulating production of regulatory cytokines IL-10 and TGF-β that dampen Th1 immune responses and NO production. Because, eosinophils exert a similar effect by producing IL-10 (Appendix C-4) and preventing local production of NO, we hypothesized that NO mediated stress on nurse cells and/or parasites impaired larval growth. We investigated the biological relevance of NO on parasite growth by infecting IL-10 deficient mice that develop strong Th1 immune responses, but we did not find a correlation between larval growth and NO levels. Other oxidative radicals may contribute to growth impairment and pharmacological inhibition studies

will be useful in addressing the roles of such mediators.

Because the skeletal muscle is the home for *T. spiralis* larvae and is an important disposal site for glycogen [24], we investigated the impact of Th2 inflammation on energy substrate uptake and parasite growth using eosinophil-deficient and STAT6-/- mice. Several studies have shown that Th1/Th2 balance impacts nutrient homeostasis in major metabolic sites. For example, using mice with macrophage-specific deletion of the peroxisome proliferator activated receptor-γ (PPARγ) that is required for maturation of M2 macrophages, it was shown that M2 macrophages are important for preventing development of insulin resistance and glucose intolerance [25, 26]. Moreover, eosinophils and STAT6 signaling reduce insulin resistance in mice supplemented with high fat diet [11, 27].

Evaluation of genes associated with nutrient homeostasis showed that AMP-activated protein kinase (AMPK) and Insulin Receptor (INSR) transcript levels were significantly increased in muscles of eosinophil-ablated mice. AMPK is a cellular regulator of energy status that has an essential role in autophagy induction, in response to starvation [28]. Autophagy is counter-regulated by the activation of the mammalian target of rapamycin (mTOR) pathway, which is associated with cell growth and proliferation [28]. To further determine if parasite growth is regulated by the AMPK/mTOR pathway, we measured transcription of genes that promote mTOR signaling, while simultaneously inhibiting AMPK activation (RAC-beta serine/threonine-protein kinase 1, 2; Akt1, Akt2). Eosinophil ablation did not impact transcription of these genes (Appendix C-2). However, because mRNA transcript levels may not necessarily correlate with enzyme activity, functional studies are imperative before ruling out the mTOR/AMPK pathway in parasite growth regulation.

Nevertheless, increased expression of both AMPK and INSR in the skeletal muscle implicate the development of moderate insulin resistance that is caused by a Th1 skewed immune response in infected muscle. However, glycogen concentrations were similar in infected muscle and the larvae isolated from the muscle of WT and ΔdblGATA mice. These results do not exclude a role for differences in muscle glucose uptake in parasite growth modulation. In particular, glycogen assay may not discriminate glucose transport variations between nurse cells of eosinophil-deficient and WT mice. The simplest approach to address this concern would be to perform qRT-PCR studies of genes associated with muscle nutrient homeostasis on infected myotubes isolated by laser capture microdissection (LCM). Enzyme activity in infected muscle and glycogen measurement in different developmental stages of muscle larvae would be essential next steps to validate PCR-based findings. Larval glycogen measurement and LCM studies exclude contaminant infiltrating cells, thus giving a more accurate readout.

Th2 immune responses may also influence other aspects of nurse cell transformation. For example, it is very well established that infected myotubes develop a vascular network and a collagen capsule, most likely to preserve larval infectivity [29, 30]. It is presumed that the nurse cell induces the formation of a vascular network (circulatory rete) to obtain host nutrients. A potent angiogenic factor, VEGF is prominent in nurse cells and infiltrating leukocytes [31]. Pro-angiogenic leukocytes, eosinophils and M2 macrophages, are numerous at sites of infection in WT mice, but are absent/ reduced in eosinophil-deficient and STAT6-/- mice [21, 32, 33]. In a preliminary experiment, we evaluated mRNA expression profiles of 84 angiogenesis-related genes using qRT-PCR array (Mouse Angiogenesis PCR Array-SAbiosciences) in diaphragm muscles recovered from infected WT and PHIL mice. At 14

dpi, several potent pro-angiogenic mediators such as fibroblast growth factor, hepatocyte growth factor, placental growth factor, matrix metalloproteinase 9, and angiopoetin 2 were upregulated in both strains of mice (AppendixA). Furthermore, plasminogen and collagen XVIII precursors for angiogenesis inhibitory molecules angiostatin [34] and endostatin [35] respectively, were selectively upregulated in PHIL mice but not in WT mice (Appendix A). G-CSF mRNA expression was also increased in tissues of eosinophil-deficient mice (Appendix A). G-CSF is a growth factor for neutrophils and neutrophil proteases mediate proteolytic degradation of SDF-1 [36], a potent chemokine that mobilizes and recruits endothelial progenitor cells to the infection microenvironment [37-39]. All of the other genes tested were similar in expression in *T. spiralis* infected WT mice. These results suggest that circulatory rete formation may be compromised in the absence of eosinophils, which may impair parasite growth by limiting access to nutrients.

The nurse cell also develops a collagen capsule [40]. Although the infected cell is known to synthesize collagen [40], it is possible that local inflammatory cells contribute to net collagen deposition. In a Th2 environment, eosinophils, fibroblasts, and macrophages produce pro-fibrotic mediators that promote collagen deposition. For example, IL-4 and IL-13 increase fibroblast chemotaxis, proliferation, and collagen synthesis [6]. In a model of allergic respiratory inflammation, eosinophil-deficient mice showed reduced collagen deposition [41].

Moreover, M2 macrophages produce Arginase-1 (Arg1) in a STAT6 dependent manner and Arg1 participates in the Krebs-Henseleit urea cycle to generate ornithine, a metabolic precursor for proline, a component of collagen [42]. We investigated the role of macrophage Arg1 in parasite growth and survival using $Arg1^{flox/flox}$;Tie2cre mice that bear Arg1 deletion in hematopoietic cells. The results showed that Arg1 did not regulate parasite growth,

but promoted parasite survival by limiting NO response. This is consistent with previous studies that showed that Arg1 competes with iNOS for arginine, thus limiting IFN-γ driven NO production in macrophages [42]. Although the results do not support a role for Arg1 in parasite growth, this does not exclude a potential role for collagen deposition on parasite establishment in the muscle. For example, there is strong evidence for Arg1-independent collagen synthesis by hematopoietic and skeletal muscle cells [43, 44]. Taken together, these results suggest that compromised angiogenesis and/or collagen deposition may be the underlying mechanisms that cause impaired larval growth. Figure 4.1b summarizes potential modulators of parasite growth as discussed in the text.

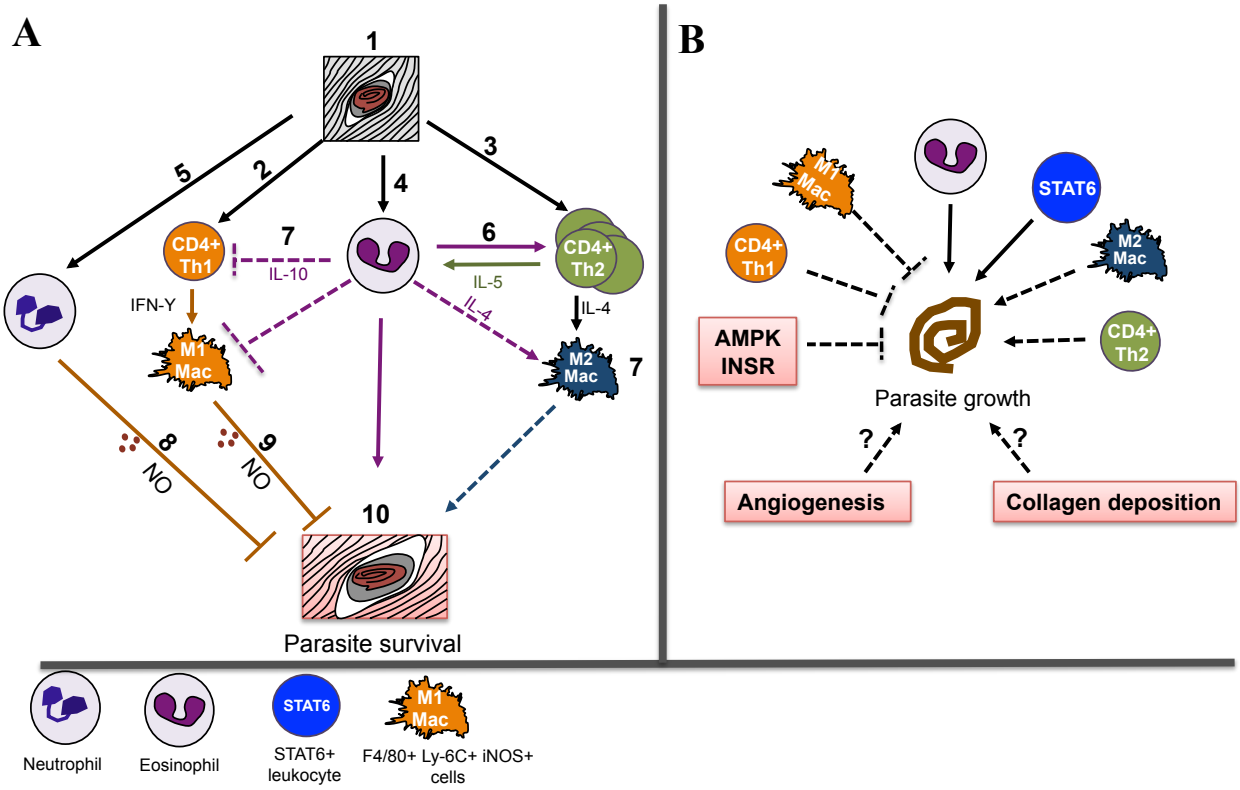

FIGURE 4.1. A, Eosinophils regulate cellular immunity and promote muscle larvae survival. Muscle infection (1) induces a mixed Th1 (2) and Th2 (3) cellular response, eosinophilia (4) and neutrophilia (5). Eosinophils limit Th1 immunity by promoting Th2 cell proliferation (6), and by producing IL-10 (7). In eosinophil ablated mice, Th1 immune responses are enhanced, and neutrophils (8) and classically activated inflammatory macrophages (9) produce NO, which clears infection. Th2 immune responses are associated with parasite survival (10). **B, Regulation of parasite growth.** Normal larval growth is dependent on eosinophils and/ or STAT6, and it is associated with Th2 cell accumulation and alternative activation of macrophages in the muscle. Parasite growth negatively correlated with Th1 immune responses, classical activation of macrophages, and AMPK and INSR transcription. Angiogenesis and collagen deposition may promote parasite growth. *Dashed lines indicate possible routes and consequences.*

In the muscle, *T. spiralis* larvae can persist for months to years, consistent with evasion or suppression of host immunity. Determining the mechanisms of immune evasion is crucial in understanding the requirements for maintenance of chronic infection. Studies with eosinophil-ablated mice suggest that the outcome of a tissue-dwelling chronic helminth infection may be to sustain an anti-inflammatory Th2 immune response, which protects the host from excessive tissue damage, while preserving parasites. Because parasite persistence may promote resistance to reinfection, it will be useful to determine if eosinophils and/or STAT6 are required to maximize host fitness during secondary infection with *T. spiralis*.

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APPENDIX-A

Mouse Angiogenesis qRT-PCR-Array on diaphragms recovered from infected WT and PHIL mice at 14dpi

A.

Gene name	Fold over uninfected	Gene name	Fold over uninfected
Brain angiogenic inhibitor 1	300	Frizzled 5	3
Tissue inhibitor of metalloproteinases 1	750	Hepatocyte growth factor	10
Matrix metalloproteinase 19	7	Midkine (neurite growth-promoting factor 2)	3.5
Integrin beta 3	6	Platelet-derived growth factor A	4
Leukocyte chemotaxin 1	2800	Placenta growth factor	10
collagen, type IV, alpha 3	19	Leptin	21
Interferon gamma	600	Matrix metalloproteinase 19	14
Chemokine (C-C motif) ligand 2	18	Thymidine phosphorylase	4.5
Chemokine (C-X-C motif) ligand 1	26	Fibroblast growth factor receptor 3	5
Chemokine (C-X-C motif) ligand 2	26	Natriuretic peptide receptor 1	4
Chemokine (C-X-C motif) ligand 5	130	Plexin domain-containing protein 1	7.5
Interleukin 6	62	TGF beta receptor 1	3.8
IL-1b	49	T-box 4	13
Tumor necrosos factor	130	eHAND	100
Sphingosine kinase 1	21	Angiopoietin 2	3.8
Transforming growth factor-alpha	24	Ephrin A1	8
Epiregulin	135	Urokinase plasminogen activator	4
Fibroblast growth factor 6	4.7		

B.

Gene name	PHIL	WT
Matriptase-2(tmprss6)	10.41	-
collagen,type XVIII, alpha	3.92	-
coagulation factor II (thrombin)	17.41	-
Granulocyte-colony stimulating factor	522.9	135
thymidine phosphorylase	13.94	-
plasminogen	19.66	-

Mouse Angiogenesis qRT-PCR-Array on diaphragms recovered from infected WT and PHIL mice at 14dpi. We evaluated mRNA expression profiles of 84 angiogenesis-related genes using qRT-PCR array (Mouse Angiogenesis PCR Array-SAbiosciences) in infected WT and PHIL diaphragm. Columns represent fold changes of individual gene expression over uninfected WT. (A) Results reported are genes whose enhanced expressions are similar between PHIL and WT. (B) All genes that are differentially expressed between WT and PHIL mice at 14dpi. Results were not confirmed using other methods. Fold differences are over uninfected WT.

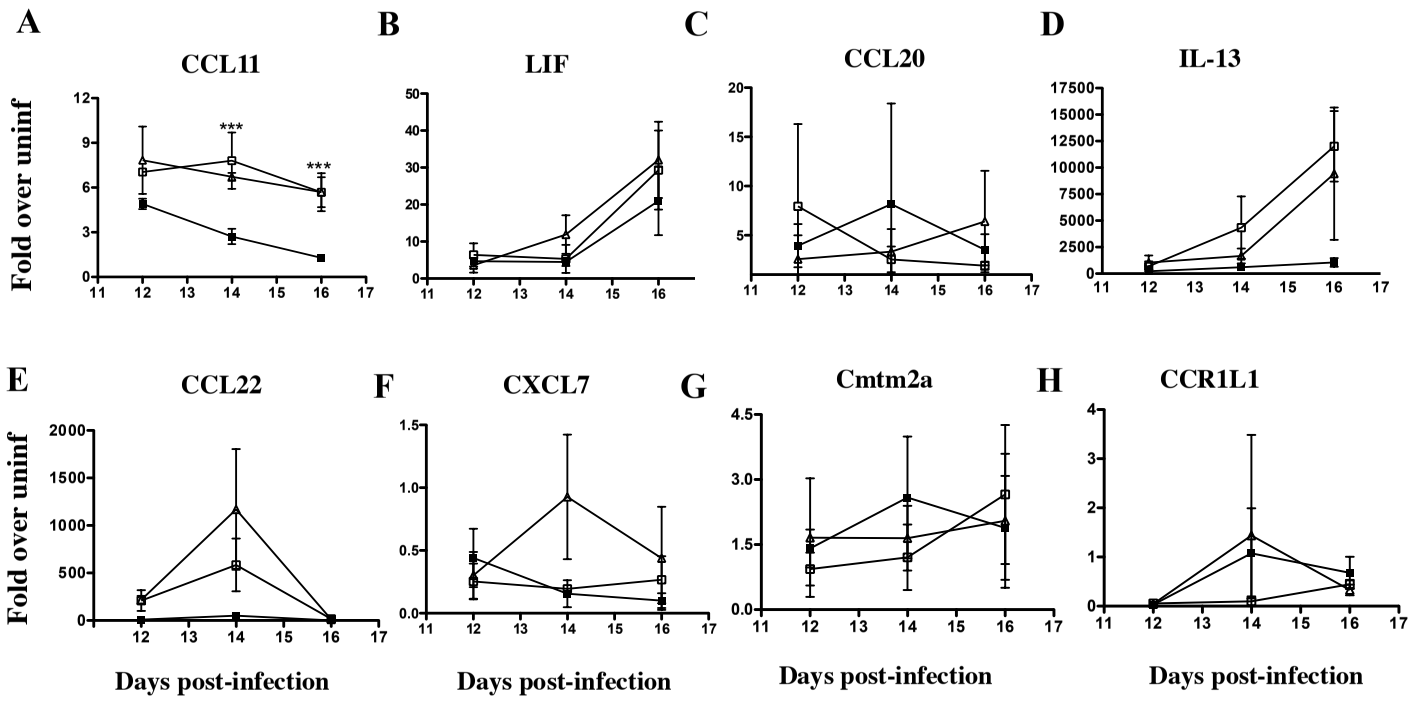
APPENDIX-B

Mouse chemokines and receptors qRT-PCR-Array on diaphragms recovered from infected WT and ΔdblGATA mice at 14dpi

Gene name	Fold Regulation
Ccl11	2.0976
Ccr3	2.0491
Cxcl13	3.284
Cxcl5	2.5425
Il13	3.0514
Ccl20	-2.5799
Ccr1l1	-5.3456
Cmtm2a	-3.106
Csf1	-2.0972
Cxcl15	-2.392
Cxcl7	-2.159
Il8ra	-5.7671
Il8rb	-4.8775
Lif	-2.4759
Trem1	-5.5306

Appendix B-1. Mouse chemokines and receptors qRT-PCR-Array on diaphragms recovered from infected WT and ΔdblGATA mice at 14dpi.

We evaluated mRNA expression profiles of 84 mouse chemokine and chemokine receptor genes using qRT-PCR array (Mouse Chemokines and Receptors PCR Array-SAbiosciences) in infected WT and ΔdblGATA diaphragm. Columns represent fold changes of individual gene expression in infected ΔdblGATA mice over infected WT. The table shows all genes that are differentially expressed between WT and ΔdblGATA mice at 14dpi. Results were partially confirmed using qRT-PCR (Appendix B-2). See chapter 3 for methods.

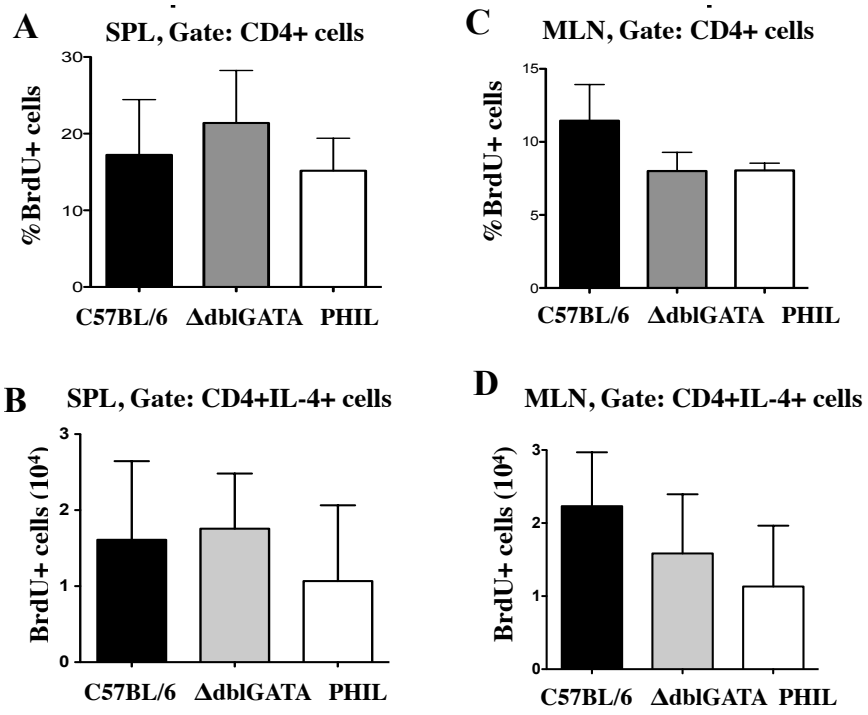


Appendix B-2. Chemokine gene expression during muscle infection with *T. spiralis*

Quantitative RT-PCR analysis of IL-13, CXCL7, LIF, CCL20, CCL22, Cmtm2a, CCR1L1 and CCL11 in muscles of PHIL, ΔdblGATA and WT mice, 12, 14 and 16 dpi. Assay was performed as described in Materials and Methods of Chapter 3. This experiment was done once, with day 14 replicated twice (Fig. 3, Chapter 3). Values represent means ± SD; n = 3–4 mice. Significant differences were determined by Student t test. ***p , 0.0001.

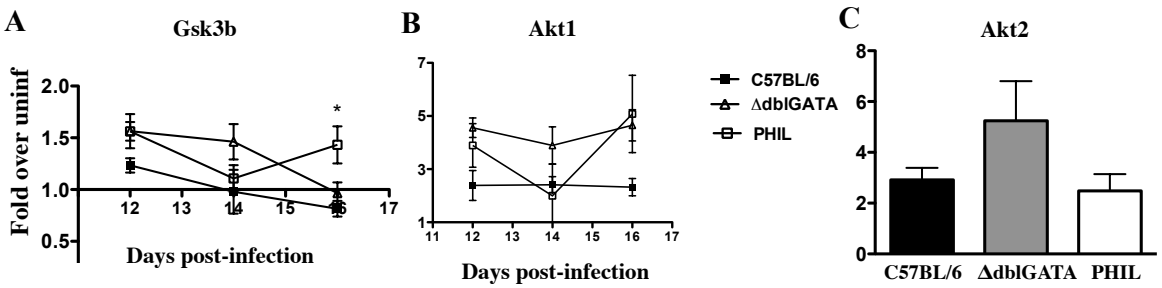
APPENDIX-C

Characterization of immune responses during muscle infection with *Trichinella spiralis*



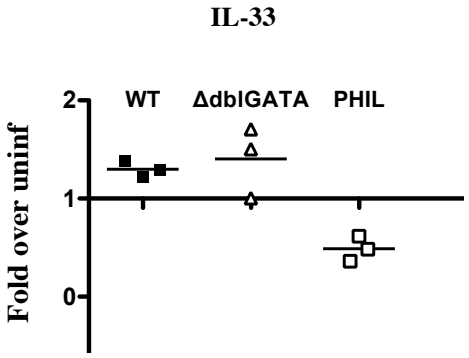
Appendix C-1. Proliferation of CD4+ population

Flow cytometry analysis of proliferating (BrdU+), CD4+ and CD4+IL-4+ cells in the spleen (*A, B*) and mesenteric lymph node (*C, D*) in infected PHIL, ΔdblGATA and WT mice at 13dpi. Results show that proliferation of CD4+ and CD4+IL-4+ cells in the SPL and MLN are similar in WT and eosinophil deficient mice. Assay was performed as described in Materials and Methods of Chapter 3. Values represent means ± SD; n = 3–4 mice. Experiments were performed twice with similar results. Differences are not significant.



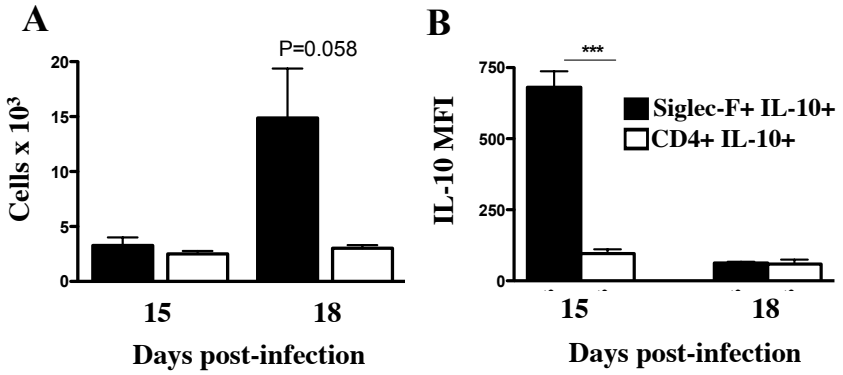
Appendix C-2. Akt1, Akt2, and Gsk3b muscle gene expression

Quantitative RT-PCR analysis of RAC-beta serine/threonine-protein kinase 1 (Akt1) and glycogen synthase kinase 3 beta (Gsk3b) in muscles of PHIL, ΔdblGATA and WT mice, at 12, 14, and 16 dpi *(A, B)* and RAC-beta serine/threonine-protein kinase 2 (Akt2) at 14dpi (*C*). There were no meaningful differences between WT and eosinophil deficient mice in expression levels of these genes that are implicated in muscle energy homeostasis. See Chapter 3 for methods. Values represent means ± SD; n = 3–4 mice. Experiments were performed twice with similar results. *p , 0.05.



Appendix C-3. IL-33 response during muscle infection

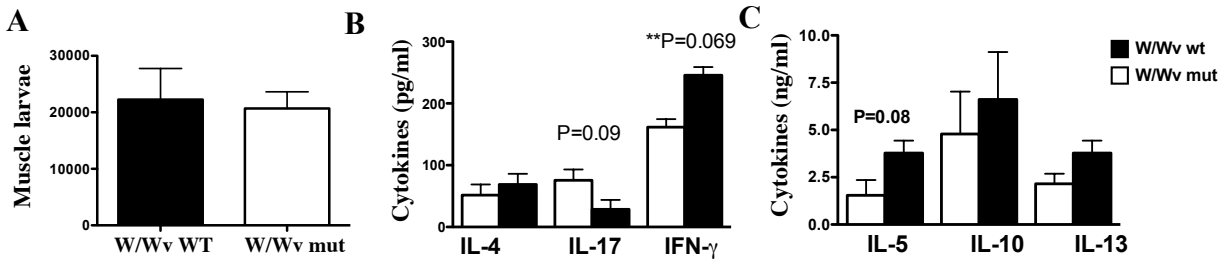
Quantitative RT-PCR analysis of IL-33 in muscles of PHIL, ΔdblGATA and WT mice, 14 dpi. Results show that infection does not significantly increase IL-33 transcription in the muscle in PHIL, ΔdblGATA and WT mice. Values represent means ± SD; n = 3–4 mice. See Chapter 3 for methods. Experiments were performed twice with similar results. Differences are not significant.



Appendix C-4. Sources of IL-10 during muscle infection

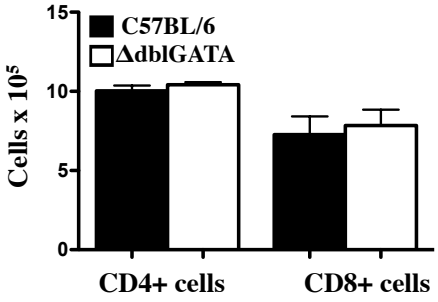
(A) Numbers of IL-10+ leukocytes and *(B)* MFI of IL-10+ leukocytes recovered from diaphragms of infected Vert-X mice. Results show that eosinophils are significant sources of IL-10 at sites of infection. See Chapter 3 for methods. Experiments were performed two times

with similar results. Values represent means ± SD; n = 3–4 mice. Significant differences were determined by Student t test. ***p , 0.0001.



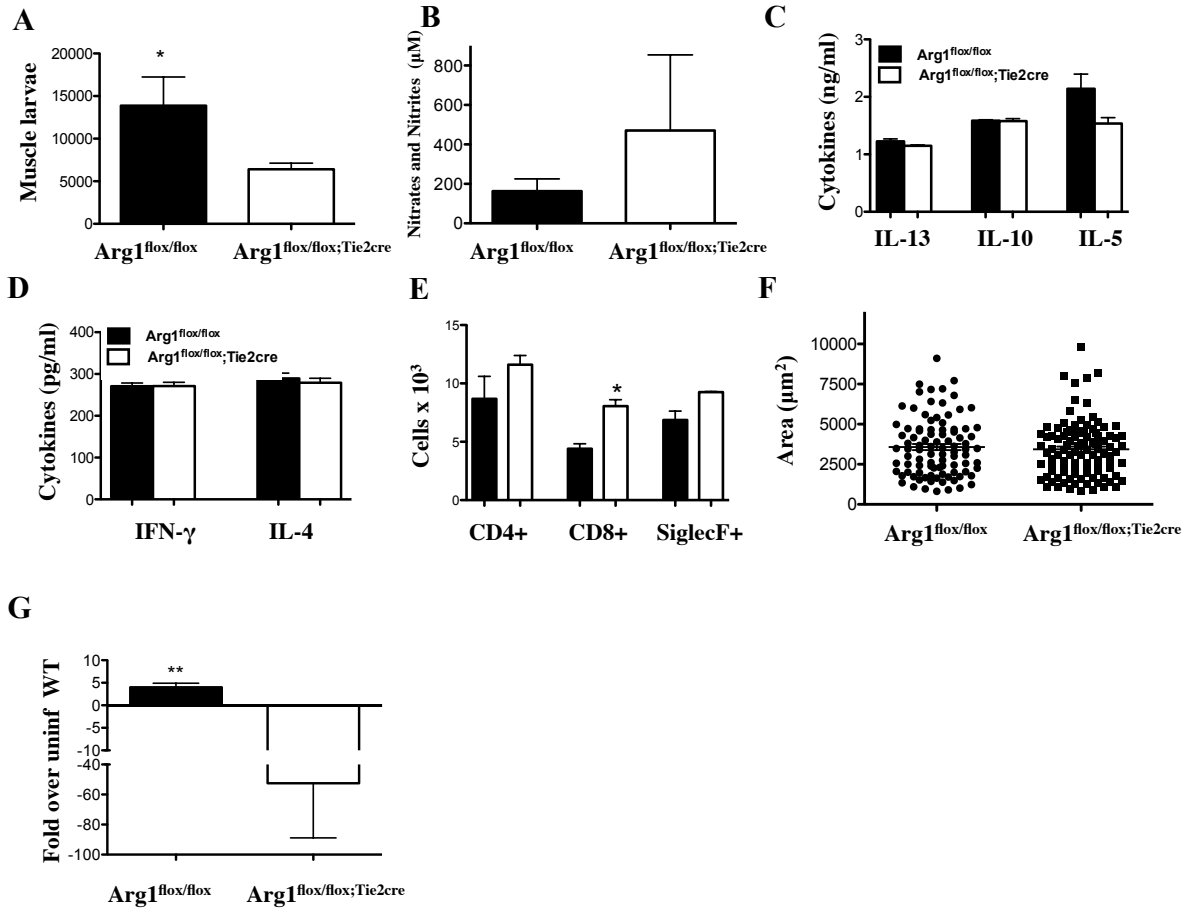
Appendix C-5. Effects of mast cells on parasite survival and immunity

(A) Larval burdens in muscles of W/Wv WT and W/Wv mut mice orally infected with 300L1, 28 dpi. *(B, C)*, Cytokines measured in Ag-stimulated cultures of CLN cells collected from W/Wv WT and W/Wv mut mice, 17 dpi. Results show that mast cell deficiency does not have a meaningful impact on cytokine response or muscle burden. See Chapter 3 for methods. Experiments were performed twice with similar results. Values represent means ± SD; n = 3–4 mice. **p , 0.001.



Appendix C-6. T cell counts in the spleen

Numbers of CD4+ and CD8+ leukocytes recovered from the spleen of infected C57BL/6 and ΔdblGATA mice, 14dpi. Results show that numbers of CD4+ and CD8+ T cells in spleen are similar between WT and ΔdblGATA mice, implying that T cells egress normally from secondary lymphoid organs in eosinophil-ablated mice. Values represent means ± SD; n = 3–4 mice. Differences are not significant.



Appendix C-7. Arg1 in parasite growth and survival.

A, Larval burdens in muscles of Arg1$^{flox/flox}$;Tie2cre and Arg1$^{flox/flox}$ mice, 26 dpi. *B,* NO end products and *(C-D)* cytokines measured in Ag-stimulated cultures of CLN cells collected from Arg1$^{flox/flox}$;Tie2cre and Arg1$^{flox/flox}$ mice, 17 dpi. *E*, CD4+, CD8+, and Siglec-F+ cells recovered from diaphragms of infected Arg1$^{flox/flox}$;Tie2cre and Arg1$^{flox/flox}$ mice, 17 dpi. *F*, Estimated area of larvae recovered from infected Arg1$^{flox/flox}$;Tie2cre and Arg1$^{flox/flox}$ mice, 17 dpi. *G*, Quantitative RT-PCR analysis of Arginase-1 (Arg1) in muscles of infected Arg1$^{flox/flox}$;Tie2cre and Arg1$^{flox/flox}$ mice, 17 dpi. Experiments were done once. Values represent means ± SD; n = 3–4 mice. *p , 0.05.